Developmental gene regulation by an ancient intercellular communication system in social amoebae

Dissertation

zur Erlangung des akademischen Grades

doctor rerum naturalium (Dr. rer. nat.)

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät

der Friedrich-Schiller-Universität Jena

Asma Asghar

geboren am 08. Februar 1982

aus Lahore, Pakistan

Jena, July 2012

Dekan: Faculty of Pharmacy and Biology

1. Gutachter: Prof. Dr. Thomas Winckler, Friedrich-Schiller-Universität Jena, Germany
2. Gutachter: Prof. Dr. Christian Hertweck, Friedrich-Schiller-Universität Jena, Germany
3. Gutachter: Prof. Dr. Pauline Schaap, University of Dundee, Scotland

Tag der öffentlichen Verteidigung: 05. März 2013

I dedicate this work

to

"My Loving Parents"

who taught me to recognize the value of biology and medicine from an early age

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ACA	Adenylyl cyclase A (aggregation stage adenylyl cyclase)
AEBSF	4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride
ARE-mRNA	AU rich elements containing mRNA
ATCC	American Type Culture Collection
Bp	base pair
CbfA	C-module binding factor A
CF	counting factor
cGMP	Cyclic Guanosine Monophosphate
CHX	Cycloheximide
CMF	conditioned medium factor
cPIG	cAMP pulse-induced gene
CRAC	Cytosolic Regulator of Adenylyl Cyclase
DBS	Dictybase stock
D. discoideum	*Dictyostelium discoideum*
DdCAD	*Dictyostelium discoideum* cell adhesion molecule
DdMEK1	*Dictyostelium discoideum* MAP kinase kinase
ePDE	Extracellular phosphodiesterase
ESI-MS	Electrospray ionization mass spectrometry
ESI-MS/MS	Electrospray Ionisation Tandem Mass Spectrometry
GAT	growth to aggregation transition
GBF	G-box Binding Factor
GbpC	cGMP-binding protein C
GCA	Guanylyl cyclase A
GCs	Guanylyl cyclases
GDT	growth to development transition
GEF	guanine nucleotide exchange factor
GloRE	Glorin Response Element
GPCRs	G-protein coupled receptors
HGT	Horizontal gene transfer
IP3	Inositol trisphosphate

ITS	Internal transcribed spacer
JNK	c-Jun N-terminal protein kinase
kDa	kilo Dalton
L	Litre
MAPK	mitogen-activated protein kinase
MAPKKK	MAP kinase kinase kinase also called MAP3K or MEKK
ml	millilitre
MQ	Milli Q, Distilled water fed through a special ion exchange cartridge to increase its purity
MW	Molecular weight
oligo (dT)	oligodesoxythymidine
PAGE	Polyacrylamide gel electrophoresis
PDE	phosphodiesterase
PDI	Phosphodiesterase Inhibitor
PH	pleckstrin homology
PKA	protein kinase A
PKA-C	Catalytic subunit of pretein kinase A
PKA-R	Regulatory subunit of pretein kinase A
PKB	protein kinase B, Akt
PKC	Protein kinase C
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol (3,4)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PLC	Phospholipase C
Pp	*Polysphondylium pallidum*
P. pallidum	*Polysphondylium pallidum*
PPM	parts per million
PSF	prestarvation factor
Ras	Rat Sarcoma (A family of proteins)
Ras-GEF	Ras- Guanine nucleotide exchange factor
RegA	Intracellular phosphodiesterase
RIP3	Ras-interacting protein
RNA-seq	RNA sequencing
Rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction

SACGB	Social Amoebas Comparative Genome Browser
SDS	sodium dodecyl sulfate
sGC	soluble guanylyl cyclase
SNP	single nucleotide polymorphism
UTR	Untranslated region

1 Introduction

The social amoebas, or *Dictyostelids*, are an excellent system to study complex communication between cells in organizing multicellular development, since they can still alternate a sophisticated programme of multicellular morphogenesis with a unicellular feeding lifestyle. Development of multicellularity requires the intricate cell-to-cell communication and coordinated regulation of numerous genes (Iranfar *et al.* 2003; Van Driessche *et al.* 2002; Williams 2006). In the present work, roles of a peptide-based cell-cell communication system in *Dictyostelids* will be studied. To introduce the theme, an overview is given of the *Dictyostelids* life history, taxonomic position, and the complex intercellular communication required at all stages of their life cycle. Then a special insight is given into the mechanism of cell-cell communication at the transition from growth to aggregation. The cAMP communication system operating at aggregation stage of *D. discoideum* is briefly introduced. The current state of research on peptide mediated cell-cell communication among *Dictyostelids* is presented and finally aims of this study and its importance are discussed.

1.1 The social amoebae- *Dictyostelids*

The *Dictyostelids*, which are called cellular slime molds, are a major group of unicellular haploid soil-dwelling microorganisms that hover at the borderline of true multicellularity (Romeralo *et al.* 2011a; Schaap *et al.* 2006). While they spend most of their life cycle as solitary amoebae, upon starvation *Dictyostelids* can aggregate to form a multicellular fruiting body that consists of dead stalk cells and live spores (Kalla *et al.* 2011). The entire process of development is coordinated by extensive *intercellular communication* through the production and in response to chemoattractants.

1.1.1 Taxonomy

For long time, since their discovery in 1869 by Brefeld (Brefeld 1869), *Dictyostelids* were considered to be “lower fungi” based on their superficial resemblance to fungi. *Dictyostelids* are also called "myxamoebae" due to the fact that single cells look like amoebae and move and feed in an amoeboid manner. In traditional systematics, all amoeba-like protists that constructed spore-bearing fruiting bodies were assigned to class Myxomycota of division Mycetozoa in the kingdom of fungi. The Myxomycota were

subdivided into the Protostelids, Myxogastrids, and the Acrasiomycetes. Acrasiomycetes included two subclasses: the *Acrasids* and the *Dictyostelids*. The *Acrasids* are significantly different from the *Dictyostelids* in the morphology of individual amoebas and aggregates and lack of cellulose in spore-bearing stalk tubes (Raper 1984; Bonner 1982).

Modern taxonomy based on a combination of cellular and molecular sequence data has revealed six supergroups of eukaryote organisms (excavates, Rhizaria, Chromalveolates, Plantae, Amoebozoa and Opisthokonts) that in turn may comprise just two superclades: unikonts (Amoebozoa, Opisthokonts) and bikonts (the other supergroups; Minge *et al.* 2009; Richards & Cavalier-Smith 2005; Keeling *et al.* 2005; Keeling 2004; Simpson & Roger 2004; Baldauf 2003; Stechmann & Cavalier-Smith 2003). *Dictyostelids* are the members of the supergroup Amoebozoa (consisting mainly of unicellular amoeba-like-organisms) that forms a sister clade to the Opisthokonts, the group containing fungi and animals, having diverged more recently than the plants (Schaap 2011b; Baldauf *et al.* 2000; Baldauf & Doolittle 1997). Obviously, morphology-based classification does not represent the genetic similarity between different groups of organisms (Schaap 2011b).

1.1.2 Phylogeny of *Dictyostelids*

Traditionally, classification of *Dictyostelids* has been based on their most notable trait, fruiting body morphology (Hagiwara 1989; Raper 1984). Based on this trend, three genera of *Dictyostelids* were defined: *Dictyostelium* (Brefeld 1869) with cellular stalks and mostly unbranched or rarely laterally branched fruiting bodies; *Polysphondylium* (Raper 1984) with regularly spaced whorls of lateral branches on cellular stalks, and *Acytostelium* (Raper 1956) which forms fruiting bodies with acellular stalks composed of cellulose.

A cladistic study by Swanson and co-workers (Swanson *et al.* 2002) proposed that traditional fruiting body morphology based classification of *Dictyostelids* is deeply flawed. Later, this suggestion was validated by the first molecular phylogeny based on alpha-tubulin and SSU (18S) rDNA sequences (Romeralo *et al.* 2007; Schaap *et al.* 2006). Molecular phylogeny constructed by Schaap *et al.* (2006; Figure 1) included nearly all of the species known at that time (most than 100 isolates of *Dictyostelids*) into four major groups. Group 1 consists of morphologically distinct *Dictyostelium* species, all of which

have small spores bearing fruiting bodies. Group 2 named “Heterostelids” includes representatives from all three of the traditional genera, accommodating all pale-colored species of *Polysphondylium* (Figure 2), at least two species of *Dictyostelium,* and all species of *Acytostelium*.

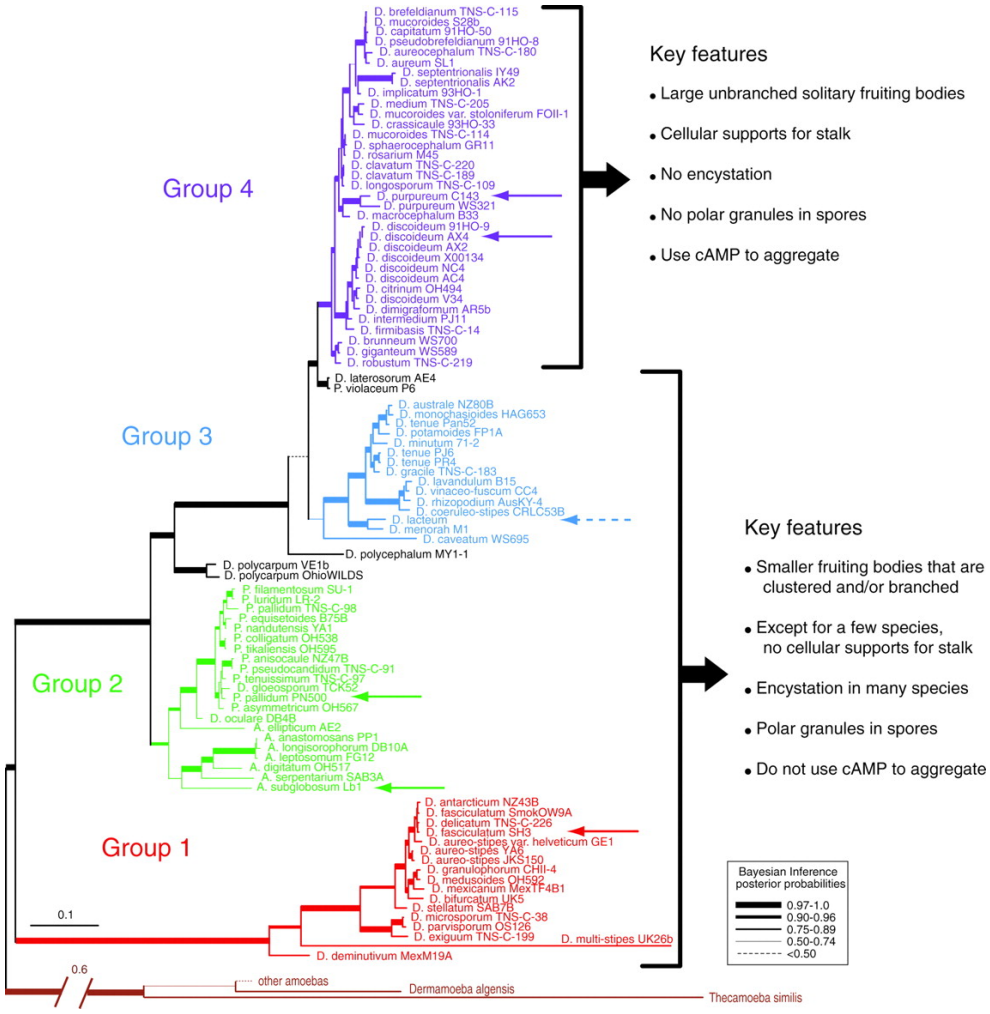


Figure 1: A global phylogeny of *Dictyostelids*. Most known species of social amoebas were subdivided into four major groups based on phylogenetic analysis of conserved SSU rDNA sequences from 1655 aligned positions using Bayesian inference. Coloured arrows refer to species with completely sequenced genomes. The family tree of social amoebas is rooted on SSU rDNA sequences of closely related solitary amoebas. (Modified from Schaap 2011a; Schaap *et al.* 2006)

Group 3 defined as “Rhizostelids” comprises a mixture of *Dictyostelium* species with rootlike support structures for their fruiting bodies and also includes the cannibalistic species, *Dictyostelium caveatum.* Group 4 is the most species-rich group of all major *Dictyostelid* groups composed almost exclusively of modern *Dictyostelium* species but

also combines a clade of two violet-colored species from two independent traditional genera, *Polysphondylium violaceum* and *Dictyostelium laterosorum* (Schaap *et al.* 2006).

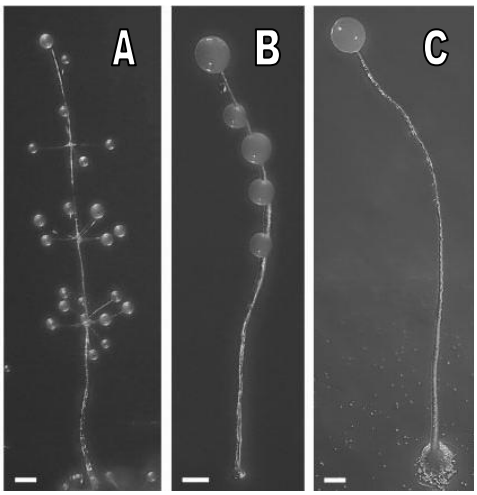


Figure 2: Fruiting body morphologies of different *Dictyostelids* species. A: *Polysphondylium pallidum* (group 2). B: *Dictyostelium rosarium* (group 4). C: *Dictyostelium discoideum* (group 4). (Modified from Schaap 2007)

Group 4 includes the best characterized *Dictyostelid* species *D. discoideum* (Figure 2), that is prominent as the simple eukaryotic model organism used to study important processes in cell biology, such as general principles for cell-to-cell communication, signal transduction, chemotaxis, cytoskeletal organization during cell motility, vesicle trafficking and phagocytosis (Schaap 2011a; Swaney *et al.* 2010; Cosson and Soldati 2008). The *D. discoideum* genome contains numerous orthologs of genes responsible for diseases in humans and has therefore become a popular model organism in biomedical research to explore the molecular basis of various human diseases, as well as the mechanism of drug action (Schaap *et al.* 2011a; Annesley & Fisher 2009; Williams *et al.* 2006; Eichinger *et al.* 2005). Studies in this organism are contributing to understand the correlation between regulated cell movement and controlled cell differentiation that gives rise to the shape and pattern during multicellular development (Schaap 2011a; Weijer 2009; Williams 2006; Kimmel & Firtel 2004).

Interestingly, none of the four molecularly defined groups are consistent with the traditional genera and none of the traditional genera are monophyletic excluding *Acytostelium* (Schaap *et al.* 2006). Moreover, first global SSU rDNA phylogeny of *Dictyostelids* revealed three lineages without clear affinity to any major group: *D.*

polycarpum (2 isolates), *D. polycephalum* (one isolate) and *D. laterosorum* + *P. violaceum* (2 isolates: Schaap *et al.* 2006).

The molecular phylogeny of social amoebae also proposed enormous molecular depth roughly comparable to that of animals but significantly greater than that of fungi. While original dictyostelid molecular phylogeny (Schaap *et al.* 2006) was based on slowly evolving SSU rDNA, it could not resolve majority of branches within the four groups. Romeralo *et al.* (2010) determined complete sequence data of more rapidly evolving internal transcribed spacer (ITS) region of rDNA for almost all species in the first molecular phylogeny that in combination with previous data of SSU rDNA sequences successfully resolved nearly all species level relationships within each of the four major groups. Five "species complexes" consisting of cryptic species (i.e. morphologically similar but genetically quite distinct species) were identified dispersed across the dictyostelid phylogeny (Romeralo *et al.* 2010; Mehdiabadi *et al.* 2009). Romeralo and colleagues (Romeralo *et al.* 2011b) reported 18S ribosomal RNA gene sequences data of new isolates identified by the Mycetozoan Global Biodiversity Survey in the past four years. Analyses of these data showed at least 50 new species and some more isolates of already characterized species scattered across the phylogentic tree, breaking up many previously isolated long branches. The resulting highly extended tree now includes eight major groups instead of the formerly recognised four (Romeralo *et al.* 2011c; Romeralo *et al.* 2011b; Schaap *et al.* 2006). Three major groups in new highly resolved phylogeny (Romeralo *et al.* 2011b) correspond to "lineages" that included only one or two sequences in the first molecular phylogeny (Schaap *et al.* 2006), and two groups originate from a firmly supported deep split in Group 2 (Romeralo *et al.* 2011b). The new groups are referred as the "*polycarpum*", "*polycephalum*" and "*violaceum*" complexes (Romeralo *et al.* 2011b) in order to maintain the original group numbering scheme as presented by Schaap *et al.* (2006) until formal names can be ascribed (Romeralo *et al.* 2011b: Schaap *et al.* 2006). The new species also broaden the known morphological variance of the four major groups defined by Schaap *et al.* (2006), challenging nearly all previously suggested deep morphological patterns (Romeralo *et al.* 2011b; Schaap *et al.* 2006).

Currently two positions are suggested for the root of *Dictyostelids,* either positioning Group 1 as the earliest lineage that diverged from a common ancestor (root 1, Figure 1, Schaap *et al.* 2006) or bifurcating the taxon approximately equally along the branch connecting Groups 1 + 2 and Groups 3 + 4 (root 2; Romeralo *et al.* 2011b; Heidel &

Glöckner 2008; Schaap *et al.* 2006). Depending upon the outgroup used, analyses of SSU rDNA and alpha-tubulin present either alternative without concrete statistical support (Schaap *et al.* 2006), however, phylogenetic analysis of mitochondrial genes (Heidel & Glöckner 2008) strongly supported root 2.

In addition to the genome of the model *D. discoideum* (Eichinger *et al.* 2005), the genomes of *Dictyostelium fasciculatum* (group 1 species) and *P. pallidum* (group 2) are now entirely sequenced (Heidel *et al.* 2011). Draft sequence data for *Dictyostelium purpureum* (group 4; Sucgang *et al.* 2011) are also accessible and the genome of a group 3 species *Dictyostelium lacteum* is presently being assembled. Genome sequence of *Acytostelium subglobosum* is also available now (http://acytodb.biol.tsukuba.ac.jp/cgi-bin/info.cgi?page=1; unpublished).

1.2 Communication modules in the life cycle of *Dictyostelids*

Dictyostelids start life as individual amoeboid cells that feed on bacteria in decaying vegetation (Schaap 2011b). As cells grow and divide, they have the ability to sense the gradients of metabolites secreted by bacteria they feed on. With an increase in population, cells start to behave cooperatively and secrete quorum-sensing factors to track population size. When the switch to enter development is made, intricate *intercellular communication* through the coordinated secretion of the chemoattractant governs the number and directionality of cells to aggregate into multicellular mounds (Schaap 2011b). The top of mound continues to emit chemoattractant pulses and is pushed upwards by the displacement of cells beneath (Schapp 2011b; Siegert & Weijer, 1995). The emerging multicellular structure of cells then falls over and is now called the “slug”. Within this structure, cells respond to and trigger cues that differentiate the clonal population into the specific cell types (Mahadeo & Parent 2006). The best fed amoebae prepare to become dormant spore (Schaap 2011b), whereas the rest are destined to form different supporting regions of the terminal fruiting body (Figure 3). The slug migrates as a more or less coherent unit, with a head region searching environmental conditions favourable for the formation of fruiting bodies (Kessin 2001). The slug stage proceeds to a process called culmination, which involves the terminal differentiation of the multicelluar organism to build fruiting body (Figure 3).

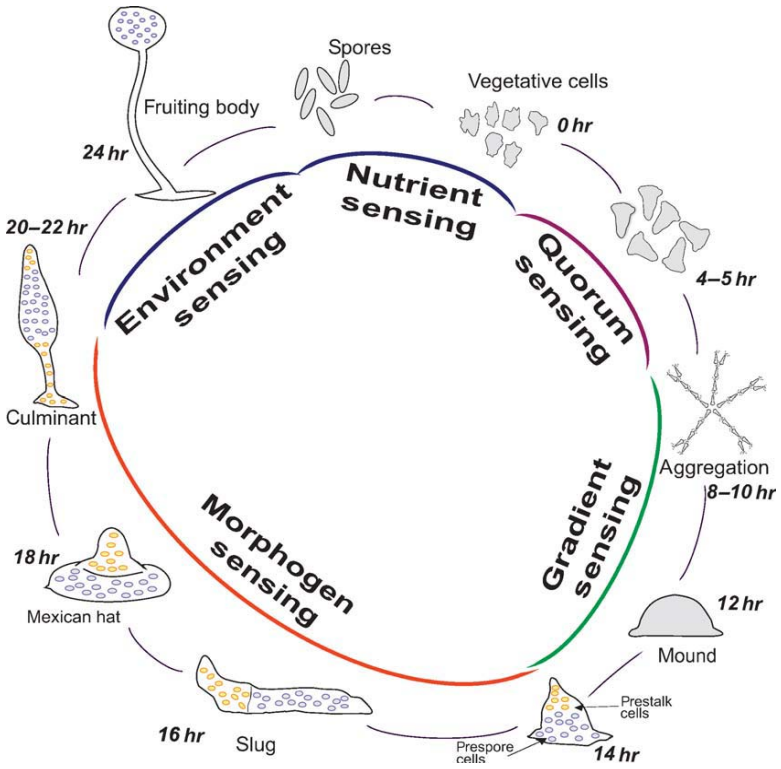


Figure 3: Schematic illustration of the 24 hour life cycle of *Dictyostelium* divided into five signalling modules. Time after starvation (hours) is marked next to each stage of development. Complex cell-cell communication is required at all these developmental check points (Mahadeo & Parent 2006).

Depending on the species, fruiting bodies differ widely in morphology but essentially consist of one or more stalks, composed of vacuolated dead cells bearing aloft one or more distinct spore masses in a variety of arrangements (Raper 1984). The spores have the ability to sense the environment (Kishi *et al.* 1998) and cues from each other to control the decision to germinate (Mahadeo & Parent 2006). Once the germination process has begun, spores can again communicate with each other to release amoebae in a synchronous fashion (Cotter *et al.* 2000).

1.2.1 Communication at the Transition from Growth to Aggregation

The life cycle of *Dictyostelids* is marked by two completely independent phases: growth and development. During growth period unicellular amoebae feed on bacteria and multiply by binary fission. Upon starvation, cells stop dividing and initiate a differentiation programme leading to the erection of a fruiting body (Kessin 2001; Romeralo *et al.*

2011b; Raper 1940; Bonner 1967). The transition between these two phases occurs through a process of *aggregation*, during which the single amoebae gather to form a multicellular mound which subsequently differentiates into a migrating slug. The well-coordinated activity of a large number of cells engaged in aggregation is mediated by extensive *intercellular communication* that involves the production of concentration gradients of diffusible chemoattractant molecules known as *"acrasins"*, and the reading of the gradient at closely determined intervals of time (Konijn *et al.* 1967; Bonner 1947). Because traditionally *Dictyostelids* were placed together with *Acrasids* in the subdivision Acrasiomycetes of class Myxomycota, the term '*acrasin'* was proposed by John Bonner to define " a type of substance consisting either of one or numerous compounds which is responsible for stimulating and directing aggregation in certain members of the Acrasiales" (Bonner 1947).

The 'acrasins' underlying aggregation in some *Dictyostelid* species have been identified. Model species *D. discoideum* and other investigated group four species use cAMP as acrasin (Schaap *et al.* 2006; Konijn *et al.*1969; Barkley 1969; Konijn *et al.* 1968; Konijn *et al.* 1967), *Dictyostelium minutum* utilizes folic acid derivative (De Wit & Konijn 1983), *Dictyostelium lacteum* a pterin derivative (Van Haastert *et al.* 1982) and *Polysphondylium violaceum* and *Dictyostelium caveatum* use a modified dipeptide glorin (Shimomura *et al.* 1982; Waddell 1982b) to aggregate. A fascinating aspect of *D. discoideum* and other group 4 species development is that secreted cAMP is used both as a chemoattractant to coordinate aggregation and as a morphogen in organizing the construction of fruiting bodies. In comparison, in group 1-3 *Dictyostelids*, extracellular cAMP exerts its effects during postaggregative stages as a morphogen while the aggregative chemoattractant may be different.

For successful aggregation, starving cells must develop a number of specialized properties in the pre-aggregation phase which separates the growth and aggregation phases (Kessin 2001). In a starving population, a few autonomous cells must appear occasionally that start to release *acrasin* spontaneously, thereby initiating the coordinated activity of surrounding cells (Shaffer 1962). The amoebae must be able to detect the *acrasin* and respond by moving up its gradient towards increasing concentration. After being stimulated by *acrasin*, the starving amoebae must develop the capacity to synthesize and release the same by themselves, thus propagating the *acrasin* signal to neighboring cells. Also, cells must acquire the ability to become refractory to further *acrasin* stimuli for a short period of time (Brzostowski & Kimmel 2006;

Kessin 2001; Cohen & Robertson 1971; Gerisch 1968; Shaffer 1957b). Cells must also be capable to remove *acrasin* locally by acrasinase activity so that acrasin concentration in the immediate vicinity of cells is insufficient to re-excite them as they come out of the refractory period (Shaffer 1957a). After individual starving cells have developed aggregation competence, a coordinated wave of activity begins at the autonomous cell resulting in secretion of *acrasin* that marks the beginning of the aggregation phase.

1.2.2 Receptors for cell communication in *Dictyostelids*

Social amoebae use different chemical signals for cell-cell communication during all phases of their life cycle. Accordingly, these organisms express stage-specific cell-surface receptors that distinctively bind the signal molecules, thereby discriminating between similar compounds in the soup of competing molecules. During growth phase, *Dictyostelids* can sense the folates emitting bacterial food, using highly specific folate receptors on their cell surface membranes (Pan *et al.* 1972; Wurster & Butz 1980; Van Driel 1981; Tillinghast & Newell 1984). When the food source is exhausted, amoebae develop different receptors to detect small communicating molecules, i.e. 'acrasins' that coordinate aggregation of amoebae to form a multicellular organism. The molecules that mediate aggregation, and the receptors involved, differ from species to species.

The convoluted mechanism of *intercellular communication* intervening aggregation is well determined in the model organism *D. discoideum* that uses cAMP as aggregative signalling molecule, i.e. *acrasin,* but the details of multicellular development are largely unknown in other species (Bonner 2009; Alvarez-Curto *et al.* 2005; Kessin 2001).

1.2.3 Previous knowledge of cell-cell communication in *D. discoideum*

1.2.3.1 Secreted factors that regulate gene expression in *Dictyostelids*

In *D. discoideum, growth to aggregation transition* is initially regulated by quorum sensing signal molecules including prestarvation factor (PSF), conditioned medium factor (CMF), and counting factor (CF). PSF, a glycoprotein (Mahadeo and Parent 2006; Clarke *et al.* 1988; Burns *et al.* 1981) is constantly secreted by growing cells to monitor their cell density relative to that of their bacterial food source. When the ratio of PSF relative to that of bacteria rises above a certain threshold, cells stop growing and PSF initiates to 'prime' the cells for the upcoming process of aggregation by inducing the expression of

several early developmental genes (Schaap 2011a; Clarke & Gomer 1995). First genes induced by PSF include those encoding discoidins and lectins, proteins that play a role in cytoskeletal organization and cell morphology during aggregation (Rathi & Clarke 1992; Rathi *et al.* 1991). One of the critical signalling pathways modulating the *growth to aggregation transition* involves the activation of the serine/threonine kinase YakA by the PSF response pathway (Schaap 2011b; Souza *et al.* 1998). Activation of YakA leads to the phosphorylation and inhibition of PufA, a negative regulator of translation. In feeding cells of *D. discoideum*, PufA blocks the translation of mRNA encoding PkaC, the catalytic subunit of pretein kinase A (PKA) by binding to the 3' untranslated region (UTR) of PkaC mRNA (Schaap 2011b; Souza *et al.* 1999). PkaC modulates the timing of early developmental events by regulating the expression of vital cAMP signaling proteins such as cAR1 and ACA (Anjard *et al.* 1992; Mann *et al.* 1992). PKA activity is not required for growth, but it is a compulsion for the transition from growth to aggregation (Schaap 2011a; Schulkes & Schaap 1995; Simon *et al.* 1989). At the onset of starvation, secretion of PSF declines rapidly (Rathi *et al.* 1991). A second glycoprotein, CMF (conditioned medium factor), is secreted during starvation and induces initial expression of genes whose products are required for cAMP relay system, which is necessary for the cells to aggregate (Yuen *et al.* 1995; van Haastert *et al.* 1996; Brazill *et al.* 1998). CMF acts by binding to a developmentally regulated cell surface CMF receptor (Jain & Gomer 1994). PSF- and CMF-induced genes include adenylyl cyclase A *'acaA'* responsible for synthesizing cAMP*;* cAMP receptor '*cAR1*' to detect cAMP during aggregation and the extracellular phosphodiesterase '*PdsA'* for hydrolyzing cAMP (Schulkes & Schaap 1995). cAR1, PdsA and ACA together with PKA and RegA, an intracellular cAMP phosphodiesterase, form complex biochemical connections that produce cAMP pulses in an oscillatory manner (Schaap 2011a; Iranfar *et al.* 2003; Laub & Loomis 1998).

1.2.3.2 The Signalling pathways by which cAMP regulates expression of early genes

Initially a few amoebae behaving as the aggregation centres start to emit periodic cAMP pulses, which are detected by the surrounding amoebae via cell surface cAMP receptors (Goldbeter 2006; Kimmel & Parent 2003; Halloy *et al.* 1998; Waddell 1982a). In *D. discoideum,* the extracellular cAMP signal is detected by four seven-transmembrane G protein-coupled receptors (GPCRs), designated cAR1-4. First cAMP receptor expressed during development is cAR1, followed sequentially by cAR3, cAR2, and then cAR4. The high affinity cAMP receptors cAR1 and cAR3 are required for aggregation (Johnson *et al.*

1993, 1991; Saxe *et al.* 1991a; Sun & Devreotes 1991; Klein *et al.* 1988), however, cAR1 is the principle mediator of the effects of extracellular cAMP during early development. In *D. discoideum*, heterotrimeric G-protein complexes may include 1 of 11 α subunits coupled to a single ßγ subunit (Mahadeo & Parent 2006; Zhang *et al.* 2001; Wu *et al.* 1995a; Lilly *et al.* 1993). cAR1, the aggregation specific receptor is coupled to Gα2ßγ complex (Kumagai *et al.* 1991).

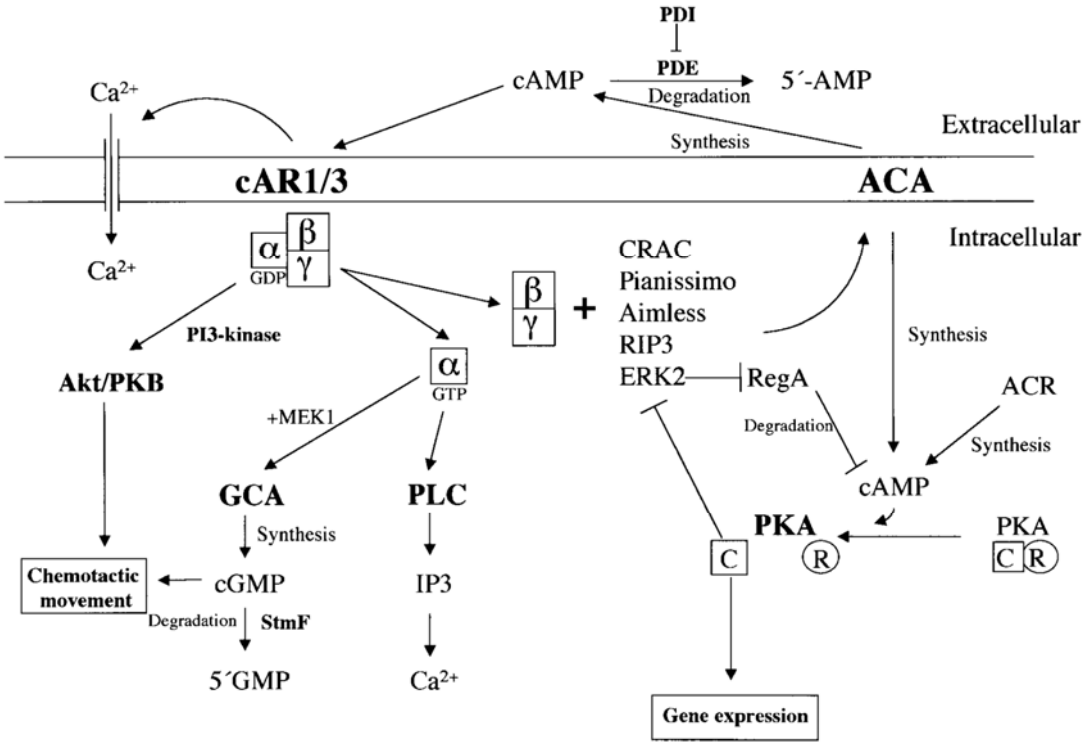


Figure 4: Signal transduction pathways modulating chemotaxis and aggregation in *Dictyostelium* (Escalante & Vicente 2000)

cAMP binding to the cAR1 transduces signal into the cells via G-protein-dependent and -independent pathways that elicit several responses associated with chemotaxis and aggregation. G-protein independent effects include calcium influx (Milne *et al.* 1995), tyrosine phosphorylation of the mitogen-activated protein kinase ERK2 (Brzostowski & Kimmel 2006; Maeda *et al.* 1996; Segall *et al.* 1995), receptor phosphorylation and post aggregative gene expression mediated by the G-box binding factor (GBF) transcription factor (Escalante & Vicente 2000; Schnitzler *et al.* 1995).

Interaction of cAMP with cAR1 initiates G-protein dependent cascade of events with the exchange of GDP for GTP in the Gα2 subunit resulting in its dissociation from ßγ subunit (Figure 4). Rapid and transient *activation of Ras* proteins, including RasG, in response to chemoattractant stimulation results in *activation of PI3K* (Phosphatidylinositol 3-kinase) that phosphorylates PIP2 (Phosphatidylinositol (3,4)-bisphosphate) to form PIP3 (Phosphatidylinositol (3,4,5)-trisphosphate; Sasaki *et al.* 2004).

The accumulation of PIP3 in the plasma membrane allows for proteins with pleckstrin homology (PH) domains to be recruited to the plasma membrane and subsequently activated. PKB (protein kinase B, Akt) and CRAC (cytosolic regulator of adenylyl cyclase) are PH domain containing proteins that become activated upon cAMP stimulation (Funamoto *et al.* 2001; Meili *et al.* 2000; Lilly & Devreotes, 1994). One of the most critical responses regulated by cAR1 is the *activation of the 12-transmembrane adenylyl cyclase* (ACA) mediated by the ßγ subunit complex (Wu *et al.* 1995a) and two cytoplasmic proteins: CRAC (Cytosolic Regulator of Adenylyl Cyclase) and Pianissimo (Insall *et al.* 1994; Chen *et al.* 1997). As shown in Figure 4, CRAC is transiently translocated to the membrane (Comer *et al.* 2005) within seconds after cAMP binding to receptor resulting in activation of ACA leading to the production and secretion of cAMP (Roos *et al.* 1975). This cAMP-induced cAMP secretion, also called cAMP relay, is critical for the outward propagation of the cAMP signal throughout the cell population. Other components necessary for receptor mediated activation of ACA include MAP Kinase ERK2 (Segall *et al.* 1995), Aimless, a Ras-guanine nucleotide exchange factor (GEF; Insall *et al.* 1996) and a novel Ras-interacting protein, RIP3 (Lee *et al.* 1999). To secrete cAMP in an oscillatory manner, the cAMP relay pathway must adapt rapidly (Parent & Devreotes, 1996) so that the adenylyl cyclase (ACA) can not be further activated until extracellular cAMP has been degraded by a secreted or membrane bound phosphodiesterase (PDE; McMains *et al.* 2008; Palsson *et al.* 1997; Hall *et al.* 1993; Franke *et al.* 1991; Podgorski *et al.* 1988; Roos *et al.* 1975; Gerisch *et al.* 1972). The cells recover sensitivity in a few minutes and these cycles of refractory and responsive states regulate the pulses of extracellular cAMP emitted with a periodicity of 6-7 minutes for several hours (Maeda *et al.* 2004). The activity of the extracellular phosphodiesterase (ePDE) is regulated by an ePDE inhibitor called PDI (Riedel *et al.* 1972). The diffusible PDI is secreted as cells arrive at the stationary growth phase (Franke *et al.* 1991; Riedel *et al.* 1972). Expression of PDI is regulated by the levels of extracellular cAMP, thus cells can control the activity of ePDE by monitoring cAMP concentrations (Franke *et al.*1991).

It is remarkable that cAMP is not only the intercellular communication agent in *Dictyostelium* but it serves also as an intracellular secondary messenger modulating different developmental signalling pathways. A fraction of the cAMP produced by the ACA accumulates within the cell to activate the cAMP-dependent protein kinase A (PKA), the main transducer of intracellular cAMP. PKA is a highly conserved protein kinase that affects activity of a variety of proteins by phosphorylation (Loomis 1998). This ubiquitous enzyme is held in an inactive form by association of the catalytic subunit (PKAC) with an inhibitory regulatory subunit (PKA-R; Funamoto *et al.* 2003; Mann *et al.* 1997; Mann *et al.* 1994; Hopper *et al.* 1993a; Harwood *et al.* 1992a; Mann *et al.* 1992; Burki *et al.* 1991; Firtel & Chapman 1990; Veron *et al.* 1988; Abe & Yanagisawa 1983). When the intracellular cAMP binds the regulatory subunit of PKA, the catalytic subunit is released, causing differential expression of developmentally regulated genes (Funamoto *et al.* 2003).

The mitogen-activated protein kinases (MAPKs) are a family of highly conserved serine/threonine kinases activated upon phosphorylation of both the threonine and tyrosine residue in the conserved TXY motif and are associated with many types of signalling pathways (Hadwiger & Nguyen 2011). *D. discoideum* ERK2 (extracellular signal related kinase 2) is a MAP kinase that is rapidly and transiently activated by extracellular cAMP acting through cAR1, involving, in part, phosphorylation by Ras proteins and/or an unidentified ERK2 kinase (Kimmel *et al.* 2004; Kimmel & Parent 2003; Wang & Segall 1998; Maeda & Firtel 1997; Aubry *et al.* 1997; Kosaka & Pears 1997; Knetsch *et al.* 1996). ERK2 inhibits activity of intracellular RegA by phosphorylating it at Thr^{676}, thereby increasing intracellular cAMP levels, which, in turn, activate PKA (Hadwiger & Nguyen 2011; McMains *et al.* 2008; Brzostowski & Kimmel 2006; Maeda *et al.* 2004). As cAMP accumulates, it is also secreted, thus recruiting neighboring cells to contribute to cAMP signal relay during aggregation (Maeda *et al.* 2004). Another putative ERK2 substrate, EPPA has been identified as being necessary for chemotaxis to cAMP and intracellular production of cAMP (Chen & Segall 2006). It has been shown by different research groups that ERK2 functions at independent stages during *Dictyostelium* development: during aggregation, ERK2 is required for the cAMP signal relay and normal chemotactic response but is not essential for aggregation-stage, cAMP pulse-induced gene expression, or for the expression of postaggregative genes. However, during multicellular development, ERK2 is required for morphogenesis and cell-type-specific gene expression (Sawai *et al.* 2005; Maeda *et al.* 2004; Zhang *et al.* 2003; Gaskins *et al.* 1996; Segall *et al.* 1995).

Previously, Loomis and colleagues developed a computational oscillatory loop model by using only six components: cAR1, ACA, ERK2, PKA, PDE and RegA (Maeda *et al.* 2004; Laub & Loomis 1998). In short, upon ligand binding, cAR1 activates both ACA and ERK2 leading to secretion of cAMP outside the cell and accumulation of cAMP inside. Extracellular PDE destroys cAMP outside the cell, while activation of PKA inside the cell blocks ACA and ERK2 activity, allowing RegA to degrade the intracellularly accumulated cAMP. This model also proposed that activated PKA may either directly or indirectly phosphorylate the receptor cAR1 causing loss of ligand binding. When the levels of intracellular cAMP are declined sufficiently by the activity of RegA, PKA is inhibited and protein phosphatases return cAR1 to its basal state and the whole process repeats thereby generating oscillations (Maeda *et al.* 2004; Escalante & Vicente 2000; Laub & Loomis 1998). However, *erkB*$^-$ and *regA*$^-$ mutants exhibit wave patterns suggestive of the spontaneous oscillations during *Dictyostelium* aggregation, therefore, Sawai *et al.* (2005) consummated that periodic cAMP signaling required the activity of the secreted cAMP phosphodiesterase for ligand clearing and not an intracellular feedback loop regulated by PKA (Sawai *et al.* 2005). Recently Brzostowski and Kimmel (2006) showed that pathways regulating ERK2 inhibition work independent of PKA activation (Brzostowski & Kimmel 2006).

cGMP is synthesized via guanylyl cyclases (Wedel & Garbers 2001) and regulate enzyme activities mainly via cGMP-dependent protein kinases (Roelofs & Van Haastert 2002; Lohmann *et al.* 1997). *Dictyostelium* contains two genes that encode for unusual guanylyl cyclase (GC) enzymes, guanylyl cyclase A (GCA; expressed mainly during growth and multicellular development; Roelofs *et al.* 2001a) and soluble guanylyl cyclase (sGC; expressed largely during cell aggregation; Veltman *et al.* 2005; Roelofs & Van Haastert 2002; Roelofs *et al.* 2001b). Extracellular cAMP binding to cAR1 activates both GCs via heterotrimeric G-proteins, leading to the transient accumulation of cGMP (Roelofs & Van Haastert 2002; Bosgraaf *et al.* 2002). In *Dictyostelium*, cGMP is implicated as one of the second messengers for chemotaxis (Veltman *et al.* 2005; van Haastert & Kuwayama 1997) that modulates the phosphorylation and localization of myosin II by binding to GbpC, a high affinity cGMP-binding protein. GbpC contains Ras, MAPKKK and Ras-GEF domains that transduce the cGMP signal, but may also function to receive other input signals (Bosgraaf *et al.* 2002; de la Roche & Cote 2001). Previously Ma *et al.* (1997) suggested that a MAP kinase kinase DdMEK1 activity is also necessary for cAMP-mediated guanylyl cyclase activation throughout the aggregation in *Dictyostelium*; nevertheless, DdMEK1 does not regulate ERK2 suggesting the presence

of independent MAP kinase cascades involved in aggregation (Sobko *et al.* 2002; Escalante & Vicente 2000; Ma *et al.*1997).

Other responses elicited by extracellular cAMP binding to cAR1 include the elevation of IP_3 levels via PLC activation mediated by heterotrimeric G-proteins (Bominaar & Haastert 1994); PLC independent IP3 formation interceded by receptor-stimulated Ca^{2+} influx (Van Dijken *et al.* 1997) and activation of the protein kinase Akt/PKB necessary for sensing and responding to the chemoattractant gradient during aggregation (Escalante & Vicente 2000; Meili *et al.* 1999).

Signal relay and chemotactic movement of several hundred thousand amoeboid cells towards cAMP source ultimately result in aggregation (Schaap 2011b; Konijn *et al.* 1967). Initially, the cells aggregate individually, but as time progresses, they form bifurcating streams by establishing head-to-tail contacts and continue to move towards the aggregation centres, eventually coalescing into hemispherical aggregates; the mounds, containing about 10^5 cells (Weijer 2004). Once aggregation is complete, the top of the mound keeps on secreting cAMP that result in continued cell movement towards the top, causing emergence of a tip (Schaap 2011a; Siegert & Weijer 1995). Tipped mound further elongates and develops into a migrating slug which proceeds through culmination to generate a fruiting body (Bonner & Lamont 2005).

Particularly relevant to this dissertation is that cAMP as an intercellular communication agent plays an important role not only in the chemotactic movement towards aggregation centres but also in the developmental regulation of gene expression, both responses being mediated via cell surface cAMP receptors. During aggregation, extracellular cAMP pulses at nM concentrations induce optimal expression of a number of aggregation-specific genes (Kessin 1988; Williams 1988; Mann & Firtel 1987; Sun & Devreotes 1991; Mann *et al.* 1988; Kimmel 1987; Chisholm *et al.* 1987; Noegel *et al.* 1986) while other genes, induced by different signals immediately with the onset of starvation, become repressed by the external cAMP signal. These differential gene expression changes cause all cells to become rapidly competent for aggregation (Schaap *et al.* 2011a; Bagorda *et al.* 2009; Maeda *et al.* 2004, Aubry & Firtel 1999; Mann *et al.* 1997; Schulkes & Schaap 1995; Gerisch *et al.* 1975). Moreover, to prevent the loss of directional information and gene expression resulting from saturation of the receptors due to excessive external cAMP, *Dictyostelium* cells use an intricate signal removal system

consisting of secreted and membrane-bound phosphodiesterases to degrade the extracellular cAMP (Gerisch *et al.* 1972; Roos *et al.* 1975).

Interestingly, *D. discoideum* cells express many developmental genes while suspended in buffer if they are stimulated with pulses of 30 nM cAMP at 6-min periods for several hours followed by high concentrations of cAMP to mimic conditions encountered on solid supports (Iranfar *et al.* 2003). Under these conditions, the cells are spatially homogeneous and express developmental genes more synchronously than when developing on solid supports where there are local variations in cell density. Additionally, this setting provides the opportunity to address the effects of cAMP pulses on gene expression more directly.

1.3 Significance of studying intercellular communication

Intercellular communication modulates a variety of biological processes in a diverse array of organisms. In mammals, precise cell-to-cell communication coordinating directed cell migration gives shape and form to developing embryos and generates many connections and interactions between the cells of nervous system during development (Dormann & Weijer, 2003). Later in life, excessive intercellular communication promotes tissue maintenance and repair, hormonal action, wound healing and directional migration of cells of our immune system from the bloodstream towards sites of infection (Wu & Lin 2011; Wang *et al.* 2011; Eccles 2004; Martin & Parkhurst, 2004). Impaired cell-cell communication is the basis for several pathological conditions including atherosclerosis, cancer metastasis, asthma, and arthritis (Braunersreuther & Mach 2006; Mrass & Weninger 2006; Eccles 2005; Charo & Taubman 2004; Trusolino & Comoglio 2002). Further insight into the molecular mechanism of cell-cell communication underlying directional sensing and cell migration is crucial for the development of treatments for these disorders, as well as in understanding normal biological processes.

1.4 Advantages of social amoebae in researching cell-cell signalling

Dictyostelids are a large ancient group of unicellular eukaryotes with an unusual multicellular stage in their life cycle (Romeralo *et al.* 2011c). Because of the simplicity of their multicellular form and rapid life cycle of just 24 hours, researching *Dictyostelids* can provide useful insight into the adaptive evolution of social behaviour and multicellularity (Heidel *et al.* 2011). A crucial part of exploring how *Dictyostelids* are able to form

multicellular bodies from their unicellular components is to understand how single cells communicate with each other in aggregation and differentiation processes. Thus *Dictyostelids* represent an ideal eukaryotic model system for studying basic principles of cell-cell communication (Das *et al.* 2011).

The factors that make *Dictyostelids* attractive as model organisms include their distinctive growth and developmental stages, relatively short and haploid life cycle and ease of growth. All known *Dictyostelids* species can be efficiently and cheaply cultured in the laboratory on bacterial lawns on agar, and many species can be grown as mass cultures in liquid media comprising glucose, peptone, and a defined mixture of vitamins and aminoacids (Schaap 2011a). It considerably simplifies the isolation and purification of cellular products for biochemical analysis and proteomics (Schaap 2011a). Complete genome sequence, about 30 Mb in size, of a few species representing three major groups (Heidel *et al.* 2011; Sucgang *et al.* 2011; Eichinger *et al.* 2005) are accessible which provide a valuable resource for the comparison of genes between species and the identification of new genes. Sequencing projects have revealed that *Dictyostelids* genomes contain many genes that are homologous to those in higher eukaryotes and are missing in other model organisms such as *Saccharomyces cerevisiae* (Heidel *et al.* 2011; Goldburg *et al.* 2006; Eichinger *et al.* 2005). Orthologs of many genes involved in human disease are found in the genome of *D. discoideum* and have been the subject of functional studies (Myre *et al.* 2011; Williams 2010; Eichinger *et al.* 2005). In future, major use of this organism seems to be in biomedical research to understand gene function and pathological mechanisms in a variety of human disorders.

Both *D. discoideum* and the group 2 species *P. pallidum* are amenable to a broad range of molecular and genetic approaches (Schaap 2011a) including random plasmid insertional mutagenesis (REMI) which facilitates the identification of mutated genes, gene disruption by homologous recombination, RNA interference (RNAi) and antisense RNAs, episomal and integrating expression plasmids, and many other techniques.

Previous work with *D. discoideum* has provided a greater insight into the components of complex intercellular communication mechanism regulating chemotaxis to cAMP during aggregation. The seven-transmembrane receptors to detect chemoattractants, components of the heterotrimeric G-proteins and many downstream signalling pathways are highly conserved between *D. discoideum* and mammalian neutrophils despite of their large evolutionary divergence (Wang *et al.* 2011; Insall 2010; Swaney *et al.* 2010; Parent

2004). This conservation not only signifies that these pathways are evolutionarily ancient, but also that research in *Dictyostelids* might be applicable to the human immune system.

Though *D. discoideum* research has contributed enormously to our exisiting knowledge of eukaryotic cell-cell communication, much work lies ahead to tackle the complexity of chemoattractant induced signalling networks regulating changes in patterns of gene expression to coordinate cell behaviour during aggregation. Therefore, researching effects mediated by acrasins other than cAMP can extend the current understanding of developmental signalling.

1.5 Previous knowledge about the acrasin of *Polysphondylium*

The polyphyletic genus *Polysphondylium* owes its individuality to the shape of the fruiting body, distinguished by the presence of whorls of side branches. Molecular phylogeny of *Dictyostelids* has placed all known *Polysphondylium species* into the ancient group 2B except *Polysphondylium violaceum* which is more related to the most derived group 4 Dictyostelids (Romeralo *et al.* 2011b; Romeralo *et al.* 2010; Schaap *et al.* 2006). It was known since the work of Shaffer (Shaffer 1953) that the acrasin of *P. violaceum* was different from that of the larger species of *Dictyostelium*. In 1976, Bonner and co-workers provided the first positive evidence that aggregation competent *P. violaceum* amoebae used an unusual dipeptide as acrasin that was later identified as N-propionyl-γ-L-glutamyl-L-ornithine-δ-lactam-ethylester, known as glorin (Figure 5; Shimomura *et al.* 1982; Wurster *et al.* 1976). Activity of glorin was found to be maximal during aggregation that declined to insignificant levels at the onset of culmination (De Wit *et al.* 1988).

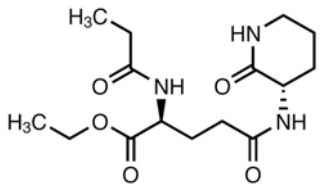


Figure 5: Structure of glorin

In addition to *Polysphondylium violaceum, Dictyostelium caveatum* (a group 3 species) and *Polysphondylium pallidum* belonging to ancient group 2 were also reported to repond chemotactically to glorin (Waddell 1982b; Shimomura *et al.* 1982; Wurster *et al.* 1976).

In *P. violaceum,* glorin signal transduction system is demonstrated to have many similarities with cAMP and folic acid transduction systems in social amoebae. Occurrence of secreted and membrane-bound acrasinases that inactivate the glorin communication signal was proposed in aggregating *P. violaceum* amoebae (Kopachik 1990; Shimomura *et al.* 1982; Wurster *et al.* 1976). It was shown that degradation of glorin by intact cells occurs rapidly (half-time 2 min) in a two-step process (De Wit *et al.* 1988). First step of this process involves hydrolyzing amide bond of the δ-lactam ring while in the second step slower cleavage of the peptide bond between propionic acid and glutamic acid takes place (Figure 6).

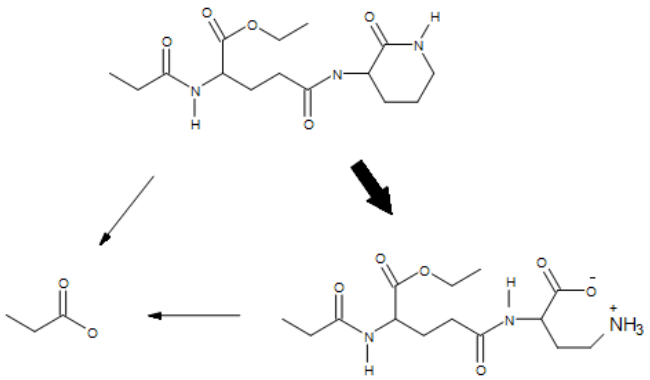


Figure 6: The chemical structures of glorin and the products of enzymatic degradation.
(Modified from De Wit *et al.* 1988).

Two-step glorin degradation may favour reuse of the building blocks. It was demonstrated that glorin degradation occurs mainly by cleavage of δ-lactam. Chemically synthesized δ-lactam-cleaved glorin, corresponding to the first product of glorin degradation, was found to be chemotactically inactive, because this product is not able to bind to receptor (De Wit *et al.* 1988). A synthetic glorin derivative in which propionic acid group was absent possessed weak chemotactic activity. Degradation of glorin by extracellular medium takes place in a similar manner as by the intact cells but at a relatively low rate (half-time 45 min). It was shown that crude homogenates also possessed some glorin inactivation activity. Cell surface glorinase activity was observed

to peak during aggregation, followed by a rapid decrease. It was noticed that cell-surface bound glorinase activity was developmentally regulated that reached its maximum during aggregation (De Wit *et al.* 1988).

Further studies to investigate the first steps in the signal transduction towards glorin suggested that in *P. violaceum* that this unusual dipeptide acts by binding to cell-surface G-protein coupled receptors (De Wit *et al.* 1988). Authors illustrated a functional interplay between cell-surface receptors and signal-transducing G-proteins by showing that binding of glorin to cell-surface receptors of aggregation-competent *P. violaceum* amoebae is modulated by guanine nucleotides, and contrarily glorin stimulus mediates the binding of GTP to cell membranes (De Wit *et al.* 1988). It was demonstrated that vegetative stage *P. violaceum* amoebae exposed sufficient number of cell-surface glorin receptors that increased slightly during aggregation, declined at later stages of development and reduced to undetectable levels during culmination (De Wit *et al.* 1988). The affinity of cell-surface glorin receptors exhibited great variations in the early hours of development but was stabilized throughout the aggregation. The receptor binding kinetic studies indicated the presence of two kinetically distinct receptors (De Wit *et al.* 1988). The same authors showed that extracellular glorin stimulus induces cGMP accumulation, a cellular response that is developmentally regulated in *P. violaceum,* peaks during aggregation and reduces to minimum at the onset of culmination (De Wit *et al.* 1988).

Oscillatory aggregation has been reported in *P. violaceum* (De Wit *et al.* 1988), but surprisingly De Wit and colleagues could not detect glorin-stimulated glorin secretion. Authors observed that glorin-induced cAMP accumulation and glorin signal relay was also absent in aggregation-competent *P. violaceum* amoebae (De Wit *et al.* 1988); therefore, they proposed that glorin is not a primary oscillator and glorin secretion during aggregation may be modulated by an unknown oscillator.

Previously, Francis (1965) suggested that in *P. pallidum* only special founder cells are capable to initiate centres in a population of starving amoebae (Francis 1965). These single cells round off and actively secrete acrasin attracting amoebae in the immediate vicinity (Konijn 1972). Such founder cells were reported to appear occasionally among aggregation sensitive amoebae of *P. violaceum* (Shaffer 1961). Francis (1965) suspected absence of any signal relay in *P. pallidum* during aggregation because he could not observe pulses of oriented cell movement among the responding amoebae (Francis 1965). *P. pallidum* cells tends to form small sized aggregates compared to large

species *D. discoideum,* therefore, Francis (1965) suggested that the concentration of acrasin in the small centres guiding formation of aggregates in *P. pallidum* may not be high enough to elicit the acrasin relay in responding amoebae (Francis 1965). Later, Jones (1976) reported that aggregation fields of *P. pallidum* amoebae display centrifugally propagated waves of excitation that are not systematically spaced (Jones 1976). In comparison, *P. violaceum* exhibited regularly spaced waves with an interval decreasing from 4 to 2.5 minutes (Jones 1976). In *P. pallidum* and *P. violaceum* wave velocities were observed to be 22 µm/min and 28 µm/min respectively (Jones 1976). Based on the observation that starving amoebae of *P. pallidum* aggregate in converging streams, he suggested that a relaying mechanism may mediate aggregation as in *D. discoideum* (Jones 1976).

It was shown by Newth & Hanna (1982) that *P. violaceum* amoebae developed in liquid suspensions gained increased ability to respond to exogenous glorin shortly after starvation before cells have acquired aggregation competency (Newth & Hanna 1982). Later, Will Kopachik (Kopachik 1990) reported some changes in protein synthesis when starving *P. violaceum* amoebae were stimulated with exogenous glorin in shaking cultures. The author showed that exogenous glorin treatment could not affect protein synthesis patterns in vegetative stage amoebae, whereas with progression of starvation, *P. violaceum* cells acquired increased sensitivity to glorin (Kopachik 1990). Furthermore, Kopachik suggested that in developing *P. violaceum* amoebae, glorin effects occur mainly during the early hours of development while sensitivity to glorin and secretion of glorin by starving cells declines rapidly once aggregation stage is over (Kopachik 1990).

Recently, Funamoto and co-workers (Funamoto *et al.* 2003) showed that *P. pallidum* cells transformed with dominant negative regulatory subunit (Rm; that can not bind cAMP) of PKA from *Dictyostelium* exhibit aggregation defects, whereas the overexpression of *Dictyostelium* PkaC in *P. pallidum* results in precocious development; demonstrating that a biochemically similar PKA mechanism works in *Dictyostelium* and *Polysphondylium* (Funamoto *et al.* 2003). The same authors proposed that PKA-requiring and a non-PKA requiring intracellular signalling pathways may regulate early developmental genes in *P. pallidum*. However, so far, glorin-mediated changes in gene expression have not been studied.

To date, little is known about the secretome of *P. pallidum* at aggregation stage of development. The availability of valuable data of *P. pallidum* PN500 genome sequence

and the advanced proteomics technology provides the foundation for the analysis of extracellular proteins secreted by aggregating *P. pallidum* cells.

1.6 Objectives of this Study

Glorin is a peptide chemoattractant used by *Polysphondylium violaceum* amoebae for cell-cell communication to coordinate the process of aggregation (Shimomura *et al.* 1982; Wurster *et al.* 1976). An ancient group 2 species *Polysphondylium pallidum* also exhibits chemotactic activity towards glorin (Shimomura *et al.* 1982). The present thesis is focused on exploring glorin-based cell-cell communication among the *Dictyostelids*. For functional genomic studies, *P. pallidum* isolate PN500 is employed owing to its genetic tractability (Heidel *et al.* 2011).

Recently, molecular phylogeny data (Schaap *et al.* 2006) suggested that glorin-based cell-cell communication might be quite wide-spread among the *Dictyostelids*. Based on this hypothesis, in the present research work the roots of glorin-mediated communication are being traced in the *Dictyostelids* phylogeny and the question will be addressed how common this peptide-based communication is in the phylogenetic descent of the social amoebae. A proteomics approach will be used to search for the putative glorin-degrading enzyme by analyzing the secretory proteome of aggregating *P. pallidum* PN500 amoebae.

P. pallidum PN500 is an emerging model organism with full experimental and genetic amenability (Schaap 2011a). It could be interesting to study if glorin-based intercellular communication also executes intracellular communication, i.e. signal transduction via binding to its cognate receptor. This possibility will be examined in the present study by researching whether glorin communication induces differential gene expression in aggregation competent *P. pallidum* amoebae. For these investigations, Illumina sequencing technology will be applied, taking advantage of the fully sequenced and annotated reference genome of *P. pallidum* PN500 (Heidel *et al.* 2011). Genome-wide analysis will be performed to determine changes in total cellular mRNA levels in response to the stimulation of *P. pallidum* cells with exogenous glorin. Changes in global gene expression in response to starvation will also be investigated in parallel. Interaction between glorin communication system and the post-aggregation cAMP signalling system of *P. pallidum* PN500 will also be scrutinized. A critical question to address is whether glorin-induced gene expression would require *de novo* protein synthesis. Finally, this study will characterize the developmental effects of ‘glorin-stimulated cell signalling’ in starving *P. pallidum* cells.

1.7 Significance of this work

The research work presented in this dissertation holds considerable promise. Firstly, it provides intriguing information about the origins of peptide communication in *Dictyostelids.* Secondly, it exquisitely compares signalling induced by two different acrasin systems (i.e. cAMP system and the glorin system) at the transition from growth to aggregation. *D. discoideum* that uses cAMP as aggregative chemoattractant and *P. pallidum*, which is presumed to use glorin as acrasin during aggregation, are similar in basic patterns of their life histories but biochemical relationship between both is utterly vague. If glorin is the acrasin of *P. pallidum*, it is expected to exert similar effects in organizing aggregation as cAMP does in *D. discoideum*. The fact that cAMP binding to the cell-surface receptors initiates distinct signaling pathways leading to differential changes in gene expression (that regulate *diverse cellular functions* during aggregation) prompted us to do comparative signal-receptor physiology with glorin. In this thesis, recent data on developmental roles of glorin mediated cell-cell signalling is presented with a particular emphasis on gene regulation by this vital cell behaviour that is suspected to coordinate aggregation in *P. pallidum*.

It has been reported that *P. pallidum* also secretes cAMP (Jones & Robertson 1976; Konijn *et al.* 1968) which has been shown by Schaap and colleagues to be involved in post-aggregation differentiation events in this species (Kawabe *et al.* 2009; Alvarez-Curto *et al.* 2005). The assumption that glorin mediates aggregation in *P. pallidum* suggests a clear chemical distinction between the chemotactic system coordinating aggregation and cAMP differentiation system. This project provides the initial insight into the topic whether post-aggregation cAMP system is induced by aggregation-specific glorin system or both systems work independently in the development of *P. pallidum.*

Structurally, glorin is an interesting molecule, it is an exceptional dipeptide and like so many peptide attractants of leukocytes, it is also terminally blocked. Though N-formylmethionyl peptides used for leukocyte chemotaxis do not attract *P. violaceum* amoebae that use glorin as chemotactic agent, but the basic molecular similarity is striking despite the difference in specificity (Shimomura *et al.* 1982; Devreotes & Zigmond 1988). Researching signalling pathways induced by this unique molecule may provide further insight into signal transduction networks that coordinate immune cell functions.

2 Material and Methods

2.1 Material

2.1.1 Laboratory equipments

Autoclave:	Varioklav Dampfsterilisator (H + P Labortechnik)
	Hiclave® HV-85L (HMC)
DNA electrophoresis:	PerfectBlue Gelsystem Mini (Peqlab)
Protein electrophoresis:	PerfectBlue Doublegel system Twin M (Peqlab)
Gel documentation:	Gel Jet Imager 2004 (Intas)
Microscope:	Binokular-Mikroskop SZX9 (Olympus)
	Mikroskop CXX 41 (Olympus)
	Lichtquelle U-RFL-T (Olympus)
	Kamera Evolution®
	MP COLOR (Media Cybernetics)
PCR machine:	Primus 96 advanced (Peqlab)
pH meter:	pH-Meter pH 526 MultiCal® (WTW)
Real-time Thermocycler:	Mx3000P (Stratagene)
Laminar Flow Hood (LAF):	MRF-B (Steag)
UV/VIS-Spectrometer:	BioPhotometer (Eppendorf)
	DU®640 Spectrophotometer (Beckman)
Vortexer:	VF2 (Janke & Kunkel, IKA Labortechnik)
Weighing machine:	E2000D, BA 6100 und PT 310 (Sartorius)
Analytical weighing machine:	A 210 P, BP 210 P und MC 210 P (Sartorius)
Water bath:	Seradest SD 2800 (SERAL Reinstwassersysteme)
	Serapur Delta (SERAL Reinstwassersysteme)
Cell counter:	CASY-Zellzähler Modell DT (Innovatis, Schärfe System)
Rotary evaporator:	Concentrator 5301 (Eppendorf)

Centrifuge machines	Centrifuge 5415 D (Eppendorf)
	Centrifuge 5810R (Eppendorf)
	J2-MC (Beckman)
	Labofuge 400R (Heraeus)
	Multifuge 1 L-R (Heraeus)
	Megafuge 1.0R (Heraeus)
Gel dryer:	Gel Dryer Model 543 (Bio-Rad)
Freeze dryer	VIRTIS

2.1.2 Chemicals

Substance	**Manufacturer**
Glorin	Phoenix Pharmaceuticals (Burlingame CA, USA)
Acrylamide	Merck
Agar-Agar	Roth
Agarose (peqGold Universal-Agarose)	Peqlab
APS	Roth
ATP	Roth
Bacto-Pepton	BD Biosciences
Bromophenolblue	Roth
BSA (Restriktionshydrolyse)	New England Biolabs
Calcium chloride dihydrate	Roth
cAMP	Roth
Chlorophorm/Isoamylalcohol	Roth
DMSO	Roth
dNTPs	Roth
D-Glucose-Monohydrat	Roth
EDTA	Roth
Ethidiumbromide	Roth
Glycerin 86 %	Roth
Glycin	Roth
Yeast extract	Serva
Potassium acetate	Roth
Potassium chloride	Roth

Potassium -di-hydrogen phosphate	Prolabo
Di-potassium hydrogen phosphate	Fluka
Laemmli-Puffer	Sigma
Magnesiumchlorid-Hexahydrate	Merck
β-Mercaptoethanol	Ferak, Berlin
Sodiumacetate trihydrat	Roth
Sodium chloride	Roth
Sodium carbonate	Roth
Sodium dihydrogen carbonate	Roth
Disodium hydrogen phosphate dihydrate	Roth
Disodium hydrogen phosphate anhydrous	Roth
Phenol-Chloroform-Isoamylalkohol (25:24:1)	Roth
Collecting gel buffer	BDH
Seperating gel buffer	Applichem
SDS	Roth
TEMED	Roth
Tris	Roth
Tween 20	Roth
Cycloheximide	Sigma
Silver nitrate	Roth
Sodium thiosulphate pentahydrate	Roth
Formaldehyde 37%	Roth
Methanol	Roth
Acetic acid	Roth

2.1.3 Solutions and Buffers

Solution/Buffer	Composition	
1X Low TE (pH 8):	Tris-HCl	10 mM
	EDTA	1 mM
PBS 10X (pH 7.2):	Na_2HPO_4	80 mM
	KH_2PO_4	15 mM

	KCl	25 mM
	NaCl	1.4 M
Phosphate buffer 50X (pH 7):	Na_2PO_4	100 mM
	KH_2PO_4	735 mM
TBE 10X (pH 8.3):	Tris	1 M
	EDTA	20 mM
	H_3BO_3	0.5 M
Tris-HCl (pH 8):	Tris	1 M
	pH value set to 8.0 with HCl	
RNase A solution:	1mg/ml in Milli-Q water	
SDS running buffer (10X):	Tris	60.4 g
	Glycin	288 g
	SDS	20 g
	2 litre	Milli-Q water
Laemmli buffer (2X):	SDS	4%
	glycerol	20%
	2-mercaptoethanol	10%
	bromphenol blue	0.004%
	Tris HCl	0.125 M
	pH approx. 6.8.	

2.1.3.1 Solutions for silver staining

Following solutions were prepared for silver staining:

Fixation solution:	Mixed together 450 ml methanol, 50 ml acetic acid and 500 ml water. The solution was stored at room

	temperature for at least 1 month.
Rinsing solution:	Distilled water 1L.
Sensitization solution:	Dissolved 0.2 gm sodium thiosulphate pentahydrate in 1 L water. This solution was prepared fresh.
Silver solution:	Dissolved 1 g silver nitrate in 1 L water. This solution was prepared fresh.
Development solution:	Dissolved 20 g sodium carbonate in 1 L water followed by addition of 1.11 ml of 37% formaldehyde. This solution was prepared fresh.
Stop solution:	Mixed together 10 ml acetic acid and 990 ml water. This solution was stored in room temperature for at least 3 months

2.1.4 Media

2.1.4.1 Liquid media

Medium	Composition	
1/5 SM medium:	Bacto-peptone	1.5 g
	Yeast extract	0.12 g
	KH_2PO_4	0.264 g
	K_2HPO_4	0.16 g
	H_2O	600 ml

Autoclaving of above medium was followed by addition of 3 ml of 40% glucose and 0.48 ml of 1 M $MgSO_4.6H_2O$

2.1.4.2 Agar media

Medium	Composition	
1/5 SM-Agar:	Agar	12 g
	1/5 SM medium	quantity sufficient to make 1 L

Autoclaving of above medium was followed by addition of 3 ml of 40% glucose and 0.48 ml of 1 M $MgSO_4.6H_2O$

2.1.5 Microorganisms

The following species were used in this study, and DBS strain numbers from the *Dictyostelium* Stock Center (http://dictybase.org/StockCenter/StockCenter.html) are given.

Dictyostelid species	Source
D. discoideum NC4	DBS0304666
D. giganteum WS589	DBS0235820
D. sphaerocephalum GR11	DBS0235889
D. oculare DB4B holotype	DBS0235852
D. gloeosporum TC-52	DBS0235825
D. fasciculatum SH3	DBS0235810
D. bifurcatum UK58	DBS0235731
D. aureo-stipes JKS5150	DBS0235725
D. parvisporum OS126	DBS0235853
D. deminutivum M19A	DBS0235744
D. microsporum Hagiwara 143	DBS0235841

P. pallidum PN500	DBS0236807
P. pallidum PN500 *tasA*$^{-}$/*tasB*$^{-}$ (Kawabe *et al.* 2009)	DBS0306505
P. pallidum CK8	DBS0236805
P. tenuissimum TNS-C-97	DBS0266526
P. tikaliensis OH595 holotype	DBS0238791
P. luridum LR-2	DBS0236804
P. pseudo-candidum Hagi-66-TNS-C-91	DBS0235858
P. asymmetricum OH567 holotype	DBS0235724
A. ellipticum AE2 holotype	DBS0235447
A. leptosonum FG12A	DBS0235449
A. subglobosum LB1	DBS0235452
P. violaceum	ATCC34156
P. pallidum WS320	ATCC44843
P. pallidum H168	a gift from M. Romeralo (University of Uppsala)

2.1.5.1 Other microorganisms

Klebsiella planticola (Source: Dicty stock centre)

2.1.6 Kits

Kit	**Manufacturer/ Supplier**
Brilliant®II SYBR® Green qRT-PCR Kit (2-Step)	Stratagene
DNeasy® Tissue Kit	Qiagen
Omniscript Reverse Transcription Kit	Qiagen
QIAquick® Gel Extraction Kit	Qiagen
QIAquick® PCR Purification Kit	Qiagen
RNase-Free DNase-Set	Qiagen
RNeasy® Mini Kit	Qiagen
Taq DNA Polymerase Kit	5’ prime

Taq DNA Polymerase Kit (Taq Pol)	Jena Bioscience

.

2.1.7 Enzymes

Enzyme	**Manufacturer/ Supplier**
Proteinase K (1 U/µl)	Fermentas
Restriktionsendonukleasen (4 – 100 U/µl)	New England BioLabs
RNase A (2 U/µl)	New England BioLabs

2.1.8 Lab materials

Lab materials	**Manufacturer/ Supplier**
1µM pore size cell culture Inserts	Becton Dickinson and company, USA
Multiwell™ 6 well plate for tissue culturing	Becton Dickinson and company, USA
Protein LoBind Tube, 1.5 ml	Eppendorf, USA
Petridishes (5 ml, 10 ml)	Greiner, Nunc
Pipette tips, unsterile	Greiner

2.1.9 Size standards

Markers	**Manufacturer/ Supplier**
DNA size standard pUC19 BsiSI (HpaII)	Jena Bioscience
DNA size standard γDNA/PstI	Fermentas
DNA size standard 0,1 kb	New England BioLabs
Molecular weight marker for SDS-PAGE (Page Ruler)	Fermentas
MagicMark ™ for SDS-PAGE	Invitrogen

2.1.10 Computer programmes

Gene Ontology Analysis Tool (GOAT) für R (Xu und Shaulsky 2005) über BioConductor, http://www.bioconductor.org/
Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/
Gene Ontology Project (GO), http://www.geneontology.org/
Dictybase (Chisholm *et al.* 2006) , http://dictybase.org/
dictyExpress (Rot *et al.* 2009), http://www.ailab.si/dictyexpress/run/
Protparam (Gasteiger *et al.* 2005) über Expasy, http://www.expasy.org/tools/protparam.html
ClustalX 2.0.11 (Thomson *et al.* 1994, Larkin *et al.* 2007), http://www.clustal.org/download/
Microsoft® Office Excel® 2003 und 2007 (Microsoft Corporation)
pDRAW 1.1, http://www.acaclone.com/
MaxPro™ QPCR Software (Stratagene)
QCapture Pro 5.0.1.26 (QImaging)
ImageJ 1.42 (http://rsbweb.nih.gov/ij/)

2.2 Methods

2.2.1 Cell biological methods

2.2.1.1 Cell culture methods

Dictyostelids were obtained from the Dictyostelium Stock Center (http://dictybase.org/StockCenter/StockCenter.html). Cells were cultured in association with *Klebsiella planticola* on 1/5 SM agar plates at $21C^0$ as described by Raper (Raper 1984). After about 45 hours of incubation, the vegetatively growing amoebae were harvested from the agar plates using cold 17 mM phosphate buffer (pH 6.2). Cells were washed free of bacteria by repeated washing with phosphate buffer (pH 6.2). $2x10^7$ cells were harvested by centrifugation and pellet was immediately stored at -80 °C for total RNA extraction or preparation of genomic DNA.

2.2.1.2 Chemotaxis assay

2.2.1.2.1 Preparation of hydrophobic agar and chemotaxis assay plates

(Ennis & Sussman 1958; Konijn & Raper 1961)

To prepare hydrophobic agar plates, agar was washed repeatedly with deionised water, suspended in 17mM phosphate buffer (pH 6.2)., dissolved by boiling and allowed to gelate. The concentration of the agar was lowered to 1% that permits amoebae to move outside the boundaries of the drop to increasing concentration gradients of acrasin after cells degrade chemoattractant in their vicinity. 1 ml of molten agar was poured in each petri plate (5 cm), agitated to allow even spreading and allowed to gelate as thin agar surface.

Synthetic glorin was obtained from Phoenix Pharmaceuticals (Burlingame CA, USA), dissolved in 17 mM phosphate buffer (pH 6.2) and stored as a 3 mM stock solution at -20 °C. Chemotaxis assays were carried out following the protocol established by Shimomura et al. (Shimomura *et al.* 1982) . Growing cells were washed to remove bacteria, adjusted to a concentration of $2x10^6$ cells/ml with 17 mM phosphate buffer (pH 6.0) and shaken for 1-5 hours at 120 rpm at 22 °C to induce aggregation competence. Cells were then concentrated by centrifugation to $2x10^8$ cells/ml and 10 µl drops of cell suspension were deposited on 1% hydrophobic phosphate agar plates containing either no acrasin or different concentrations of glorin or cAMP. In the absence of acrasin in the agar, cells stayed inside the boundaries of drops over the complete period of observation. Instead, if acrasin was embedded in the agar, extracellular acrasinase degraded the acrasin within the drop, thereby generating local acrasin gradients that caused cells to move out of the margins of the drop over a certain distance. Pictures were generally taken 3 hours after placing drops of the cells on agar surface.

2.2.1.3 Glorin pulsing experiments

Vegetatively growing *P. pallidum* cells were collected, washed, and resuspended in 17 mM phosphate buffer at a concentration of $2x10^7$cells/ml. A pellet of $2x10^7$ cells was stored immediately after washing at -80°C that served as a reference for differential gene expression in developing cells. Suspension cultures of amoebae were then shaken at 120 rpm at 22^0C. Unless otherwise specified, *P. pallidum* PN500 cells were first

prestarved for 1 hour and then treated with 1μM glorin at 10-, 20-, or 30-minute intervals for an additional 2 to 8 hours (depending on the nature of experiment). Parallel cultures were left untreated. Generally, $2x10^7$cells were collected by centrifugation 30 minutes after each pulse, pelleted and stored at -80 °C for subsequent RNA extraction.

2.2.1.4 Aggregation Analysis

2.2.1.4.1 Aggregation analysis under submerged conditions

Vegetative *P. pallidum* PN500 amoebae grown in association with *Klebsiella planticola* were harvested and washed free of bacteria four times in ice-cold 17 mM phosphate buffer (pH 6.2). The cells were then resuspended in phosphate buffer at a density of $8x10^6$cells/ml and 4 ml of this suspension was plated in monolayer on a 50-mm petri dish. Images were captured after cells have settled to the bottom of petri plates (0 hr) and when they start to aggregate.

2.2.1.4.2 Aggregation analysis on phosphate-buffered agar plates

P. pallidum PN500 amoebae were washed free of bacteria and adjusted to $2x10^7$cells/ml in cold 17 mM phosphate buffer (pH 6.2). Cells were then evenly distributed as monolayers on 50 mm phosphate-buffered agar plates at a density of $8x10^5$cells/cm^2 for development. The plates were air dried for 20 minutes and any excess of liquid was carefully removed without disturbing the cell layer. Cells were then allowed to develop at 22^{o}C. Different stages of development were observed using a stereomicroscope and images were captured at indicated time points. Cells were washed off the surface of agar plates at different stages of development, $2x10^7$cells were pelleted by centrifugation and stored at -80^{o}C for preparation of total RNA.

2.2.1.5 Development on phosphate agar to study aggregation stimulatory effects of glorin

To examine effects of glorin treatment on aggregation capacity of *P. pallidum* PN500, the suspension culture of amoebae was first prestarved for 1 hour and then distributed into three flasks (volume of the flask was 4-5 times that of the suspension) and shaken at 120 rpm. One flask was used for each of the following conditions: (i) no treatment; (ii)

pulsed glorin: pulses of glorin given every 10 minutes to a final concentration of 1µM for 2 hours; (iii) pulsed glorin: pulses of glorin given every 10 minutes to a final concentration of 100 nM for 2 hours. Cells were then washed, concentrated to $2x10^8$cells/ml by centrifugation and plated on non-nutrient agar plates at concentration of $8x10^5$ cells/cm^2. Photographs were taken when cells reached aggregation stage.

2.2.2 Molecular Biology Methods

2.2.2.1 Isolation of genomic DNA and total RNA

For isolation of total RNA or genomic DNA, a cell pellet of 2 x 10^7 cells was used, which has been stored overnight at -80 °C. The isolation of gDNA was performed using the DNeasy Tissue Kit® (Qiagen) using '*Animal cells'* protocol. For RNA isolation, the RNeasy® Mini Kit (Qiagen) was used.

2.2.2.2 cDNA synthesis

The synthesis of cDNA was carried out from 2µl of total RNA having concentration of 250 ng/µl using the Omniscript® Reverse Transcription Kit (Qiagen) and an oligo (dT)18-primer.

2.2.2.3 Quantitative RT-PCR

For the comparative expression analysis of genes, the quantitative real-time polymerase chain reaction (qRT-PCR) was performed to complementary DNAs. Gene expression levels were standardized to the gene encoding glyceraldehyde-3-phosphate dehydrogenase (gpdA, SACGB accession number PPL_12017). The *gpdA* gene was amplified using primers *gpdA*-01 and *gpdA*-02 that yielded a 218 bp PCR product from genomic DNA and a 146 bp fragment from cDNA, respectively, thereby facilitating determination of genomic DNA contaminations in cDNA preparations by conventional RT-PCR prior to real-time RT-PCR runs. The real-time quantification was performed on a **Mx3000P** (Stratagene) using the **Taq-polymerase kit** (5' primer or Jena Bioscience) together with the **Fluorescent dye EvaGreen®**. A 1:10 dilution of cDNA (synthesized as described in 2.2.2.2) was prepared and 1µl of this dilution was used in the amplification reaction mixture alongwith 300 nM final concentration of primers was used for the

amplification reactions. A list of primers used is given in Appendix Table A2. An initial denaturing step at 95℃ for 10 minutes was followed by the PCR for 40 cycles at 95℃ for 30 s, 58℃ for 30 s and 72℃ for 30 s. Subsequently, the melting curves of the resulting products were analyzed and gene expression regulation was determined by the method of Pfaffl (2001). All measurements were carried out in at least three independent experiments.

2.2.2.4 ITS sequencing (Romeralo *et al.* 2010)

A 1000 base pair (bp) fragment of ITS rDNA was amplified by PCR from genomic DNA of four isolates of *P. pallidum* using *P. pallidum* specific primers described by Romeralo et al. (Romeralo *et al.* 2007). The PCR program was composed of 5 minutes at 95 °C, followed by 30 cycles of 1 minute at 94 °C, 1 minute at 50 °C, and 2 minutes at 72 °C, and a final elongation step of 10 minutes at 72 °C. PCR products were then separated on 1% agarose gels. DNA bands excised from the gel were extracted using the QIAquick gel extraction kit. The extracted DNA was sequenced on ABI 373 sequencing machine using the same primers as were used for amplification. ITS sequences from *P. pallidum* isolates, namely, PN500, WS320, CK8, and H168 were aligned using ClustalX 2.0.11 programme. ITS sequences of *P. pallidum* PN500 (HQ213802), *P. pallidum* WS320 (HQ213803), *P. pallidum* H168 (HQ213801) and *P. pallidum* CK8 (HQ213800 were deposited in GenBank.

2.2.2.5 RNA Sequencing

P. pallidum PN500 genome sequencing project was only recently published (Heidel *et al.* 2011), therefore, facility of commercial DNA microarrays was not available. When the project presented in this dissertation was started, we had access to unpublished sequencing data of *P. pallidum* PN500 genome by courtesy of Dr. Gernot Glöckner, Fritz Lipmann Institute, Jena, Germany. To study genome-wide gene expression changes at the early stages of development, we used high-throughput Illumina RNA sequencing (RNA-seq) technology because this method produces a quantitative, digital information of all the transcripts in a given sample together with increased dynamic range and better sensitivity in contrast to hybridization based microarrays (Wang *et al.* 2009).

P. pallidum PN500 amoebae were harvested at the vegetative stage, washed free of bacteria and resuspended at a density of $2x10^7$cells/ml in cold 17mM phosphate buffer. $2x10^7$cells were stored as a pellet at -80^{o}C for total RNA prepareation. This pellet served

as “vegetative control” in RNA-seq analysis. Development was then initiated by starving amoebae for 1 hour in a suspension culture that was rotated at 120 rpm on a platform shaker at 22^{o}C. After this initial treatment, culture was divided into two parts. To examine the effects of exogenous glorin on gene expression, glorin was added at a final concentration of 1 µM at 30-min intervals for total 2 hours whereas other culture received no glorin addition and served as “control”. 2x10^{7}cells were pelleted for total RNA preparation after 1 and 2 hours of glorin treatment i.e. after 2 or 4 pulses of glorin respectively. Untreated cells were harvested at the same time points. Cells were generally harvested 30 minutes after a glorin pulse was delivered.

Transcriptome-wide expression levels were determined using Illumina/Solexa next-generation sequencing (Bentley *et al.* 2008). For library preparation an amount of 5 µg total RNA per sample was processed using Illumina’s mRNA-seq sample prep kit following the manufacturer’s instructions. The libraries were sequenced using a Genome Analyzer (GAIIx) in a single read approach with 76 cycles resulting in reads with a length of 76 nucleotides. Reads were mapped to the *P. pallidum* reference transcriptome downloaded from the Social Amoeba Comparative Genome Browser (SACGB; http://sacgb.fli-leibniz.de/cgi/index.pl) using the Illumina-supplied tool ELAND *(Bentley et al.* 2008) with standard parameters. Expression values were calculated by counting the number of unique mappable reads per transcript and normalizing these to the total number of mappable reads and length of the respective transcript (adapted from Mortazavi et al. 2008). This resulted in RPKM values (Reads Per Kilobase transcript and Million mappable reads) representing the expression level of the respective transcript. RNA-seq was performed using RNA preparations from two independent experiments. Average RPKM values derived from both experiements were used to calculate differences in gene expression. However, genes were considered as being glorin-dependently expressed only if differences in RPKM values in glorin-treated versus untreated cells were greater than 3-fold in each of the two individual RNA-seq experiments. The RNA-seq data have been deposited in the Gene Expression Omnibus data base under accession number GSE24911.

2.2.3 Biochemical methods

2.2.3.1 Proteomic analysis of secretory proteins

2.2.3.1.1 Preparation of extracellular proteins from aggregating *Polysphondylium pallidum*

To collect secreted proteins, wild-type *P. pallidum* cells were cultured in association with *Klebsiella planticola* on 1/5 SM plates at 21C^0 (Raper 1984). After about 45 hours of incubation, the vegetative amoebae were harvested from the agar plates using cold 17 mM phosphate buffer (pH 6.2) and freed of bacteria by 4 times centrifugation at 800 rpm in phosphate buffer. The final cell pellet was resuspended in 17 mM phosphate buffer at a concentration of 10^7cells/ml. Cells (10^7) were then pipetted on to a Type 353102 1 mm pore-sized polyethylene terephthalate membrane six-well format cell-culture insert (Becton Dickinson, Franklin Lakes, NJ, USA). After 10 min, the cells had settled on, and attached to, the membrane, and the buffer was gently removed from the insert. Cold 17 mM phosphate buffer containing a protease inhibitor cocktail (one tablet was used for 10 ml buffer), AEBSF to a final concentration of 0.25mM and 5 mM EDTA was added into the wells of a six-well plate. The inserts with cells were then placed in the wells. The volume of buffer in the wells was accommodated to just touch the membrane of the insert to keep the membrane and cells moist. Amoebae developed normally, with cells aggregation starting at 4 hour and branching streams formation at 8 hours (data not shown). After 8 hours of development at 21 C^0, the conditioned starvation buffer in the wells was collected in 2 ml eppendorf tubes (1 ml conditioned buffer per tube) and the samples were stored at -80 C^0.

2.2.3.1.2 Concentration of collected proteins

Frozen conditioned starvation buffer samples (1 ml sample per tube) were lyophilized and resulting powder in each tube was resuspended in 50 µl of deionized water. Samples were then immediately boiled with 50 µl of 2 x Laemmli buffer for 5 min at 95 °C to denature proteins and subsequently centrifuged for 10 min at 11000 x g in a table centrifuge (5415 D Eppendorf) to remove the large particles. Supernatant was collected and used for protein analysis by SDS-PAGE.

2.2.3.1.3 Separation of secreted proteins by SDS-PAGE

Proteins in the sample were separated by electrophoresis on 12.5 % SDS-PAGE gel according to Laemmli (1970). Seperation was done at 110- 160 Volt for 2 hours at room temperature using the 1 x SDS buffer as running buffer. For protein MW estimation, 6 µl of molecular weight marker in a range of 15 to 170 kDa (Page Ruler, Fermentas) was run alongside of the gel. Composition of gel was as follows:

Seperating gel (12.5 %):	H2O	9.2 ml
	Seperating gel buffer (4 x; pH 8.8)	7 ml
	Acrylamid (30 %)	11.7 ml
	TEMED	30 µl
	APS	90 µl
Stacking gel:	H2O	7.7 ml
	Stacking gel buffer (10 x; pH 6.8)	1 ml
	Acrylamid (30 %)	1.3 ml
	TEMED	15 µl
	APS	30 µl
SDS-Running buffer (10 x):	Tris	0.25 M
	Glycin	2 M
	SDS	1 %

2.2.3.1.4 MS- compatible silver staining of polyacrylamide gel

After electrophoresis, proteins were visualized by silver staining of polyacrylamide gel as described by Shevchenko *et al.* (1996). This staining method is mass spectrometry compatible; has no influence on peptide elution from the gel and doesn't interrupt ionization in a mass spectrometer. The limit of detection of this method is 1- 5 ng of protein per band. This technique is based on the affinity of the proteins for a cation i.e. silver. SDS also has a high affinity for silver and must be removed prior to staining by a fixation step.

After a gel run, the gel was placed in a glass tray containing 300 ml of fixation solution. The tray was shaken gently on a platform shaker for about 1 hour. After fixation, the gel

was rinsed 3 times for 30 minutes with 300 ml of MQ water on a platform shaker. Gel was then sensitized with 300 ml sensitizing solution for exactly 1.5 minutes. Rinsed the gel twice for 1 minute with plenty of MQ water and then stained with 300 ml silver solution for 30 minutes. Rinsed the gel twice for 1 minute with water, followed by development in 300 ml of “development solution” by shaking it gently until the bands acquired the desired intensity. It took almost 2-5 minutes. Development was stopped by shaking the gel gently for 5 minutes in stop solution. Care was taken to add the stopper solution slightly before the desired stain intensity was reached to get clear background. Gel was then stored in stop solution at 4 °C overnight. The next day gel was rinsed with water 2 times for 10 min and images were captured by scanning silver stained gel.

2.2.3.1.5 Mass spectrometry analysis of proteins

To identify the secreted proteins, bands were excised from the silver stained gel, cut into smaller pieces (1 × 1 mm) and sent to the mass spectrometry and proteomics core facility in the laboratory of Dr. Karl-Heinz Gührs at Leibniz Institute for Age Research, Fritz Lipmann Institute (FLI) Jena. The sample preparation procedure was based on the paper of Shevchenko *et al.* (1996) with some modifications.

2.2.3.1.5.1 ESI-MS/MS analysis

The analyses were performed by ESI-MS/MS with an LTQ Orbitrap XL instrument (Thermo Scientific) in nanospray mode. The mass spectrometer was online coupled to an HPLC (Easy nanoLC (Proxeon, Odense, Danmark)). Before analysis the sample was dissolved in 0.1 % formic acid containing 5 % acetonitrile. An aliquot of 10 µl of the sample was subjected to nano HPLC at a flow rate of 250 nl/min using an Easy nanoLC (Proxeon, Odense, Danmark) equipped with a (5 x 0.3) mm Zorbax trap column (Agilent, Waldbronn, Germany) and a 10 cm x 75 µm analytical column (NanoSeparations, Nieuwkoop, Netherlands). Separation of the components was achieved by application of a gradient from 5 % to 80 % acetonitrile in 0.1 % formic acid. The efflux was directly sprayed into the orifice of a LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Dreieich, Germany) and mass spectra were recorded. The evaluation of the spectra was performed by Xcalibur (Thermo Scientific, Dreieich, Germany).

2.2.3.1.5.2 Database search to identify proteins

The selected peptide spectra were used to search for protein candidates in the fasta database of *P. pallidum*, which was generated by ORF identification within the sequenced genome of this organism (Heidel *et al.* 2011; http://sacgb.fli-leibniz.de/cgi/index.pl) by using MASCOT (Matrix Science) software programs. The identified proteins were visualized by the software packages ProteomeDiscoverer (Thermo Scientific) and Scaffold (Proteome Software, Portland, USA). The final protein list contains only entries which are characterized by at least two independent peptides with MASCOT peptide scores above 35. Initial search parameters were set as follows: fragment tolerance: 0.80 Da (monoisotopic); parent tolerance: 10.0 PPM (monoisotopic); fixed modifications: +57 on C (carbamidomethyl); variable modifications: -14 on C (carbamyl), +16 on M (oxidation); maximum missed cleavages: 2; digestion enzyme: trypsin. Regular injections of 20 fmol of a BSA standard were used to check the current machine state. If 50 to 70 % of the protein was covered by identified peptides, machine status was taken satisfactory. Limit of sensitivity was in the range of 1 to 20 fmol protein per sample injection.

3 Results

Chemotaxis is an intriguing biological phenomenon that plays significant role during the life cycle of *Dictyostelids*. In the vegetative stage the amebae have to locate their bacterial food in the soil they dwell. At this time the amebae exhibit chemotaxis to folic acid and pterin (Liao & Kimmel 2009; Pan *et al.* 1972; Pan *et al.* 1975), both of which are secreted by bacteria, and serve as cues for nutrient localization. Cyclic AMP secreted by *E.coli* has also been shown to attract vegetative amoebae of *Dictyostelium* and *Polysphondylium* species and was suggested as efficient food-seeking mechanism (Samuel 1961; Konijn 1961; Konijn *et al.* 1967; Konijn 1969; Konijn 1972). When food source is exhausted, the amoebae gain the ability to establish a complex cell-cell communication network and aggregate to initiate social phase of their life cycle. In 1902, Olive and Potts proposed the existence of a chemical mediator of aggregation in *Dictyostelids*. Later, Bonner (1947) reported that starving amoebae release diffusible chemical agents, named 'acrasins' that are capable to orient and attract neighbouring amoebae. It has been suggested that there might be at least 8 different chemical substances prevailed as acrasins among the *Dictyostelids* (Bonner 1982). Recently, molecular phylogeny of *Dictyostelids* classified more than 100 species of social amoebae into four major groups (Schaap *et al.* 2006). The well-studied Group 4 species, *Dictyostelium discoideum,* employs the chemoattractant cAMP as communication signal to organize aggregation (Konijn *et al.* 1967). Some other group 4 species, such as *D. mucoroides, D. purpureum, and D. rosarium* have also been reported to use cAMP as acrasin (Bonner 2009; Schaap *et al.* 2006; Konijn 1973; Bonner *et al.* 1969; Konijn *et al.* 1967; Shaffer 1953). Group 3 species *D. lacteum* uses pterin as aggregation chemoattractant (Van Haastert *et al.* 1982), whereas *D. minutum* employs a derivative of folic acid (De Wit & Konijn 1983) as acrasin. A modified dipeptide glorin (N-propionyl-γ-L-glutamyl-L-ornithine-δ-lactam-ethylester) is used as cell-cell communication agent to mediate aggregation in *P. violaceum* (Shimomura *et al.* 1982), a species placed at the bottom of group 4 in the molecular phylogeny of *Dictyostelids* (Schaap *et al.* 2006). It was reported that *P. pallidum*, a group 2 species, was also responsive to glorin (Shimomura *et al.* 1982) but no detailed description of related experiments was available. *D. caveatum* (group 3 species) was described to show activity towards the acrasin of *P. violaceum* (Waddell 1982b). These previous studies provided an indication that chemical language of glorin might be widely used among the *Dictyostelids*.

Primary objective of this study was to explore how wide-spread glorin-based cell-cell communication is in the phylogenetic history of *Dictyostelids*. To address this question, commercially available glorin was used as an acrasin (or chemoattractant) and chemotactic sensitivity of a collection of *dictyostelid* species was evaluated using a bioassay described under section 3.1.

3.1 Development and validation of chemotaxis bioassay

Konijn (1961, 1968, 1969, 1972) developed an effective small population assay to test chemotaxis of *Dictyostelium* and *Polysphondylium* species to cAMP. This assay makes use of aggregation competent cells that are highly sensitive to acrasin gradients. Futrelle *et al.* (1982) showed that starving amoebae start to gain chemotactic competence within an hour after the initiation of starvation that increases gradually, such that cells acquire full competence when aggregation begins (Futrelle *et al.* 1982). Small population assay was later modified by Shimomura and colleagues to determine the chemotactic activity of *P. violaceum* to glorin that was collected from aggregating amoebae of this species (Shimomura *et al.* 1982). It has been shown that aggregation competent *P. violaceum* cells possess high number of cell surface receptors for the detection of the extracellular glorin and are capable to degrade dipeptide chemoattractant by secreting or exposing glorin degradation enzymes (De Wit *et al.* 1988).

Small population assay requires a hydrophobic agar surface of low rigidity. To prepare hydrophobic agar plates, agar is washed repeatedly with deionised water, suspended in phosphate buffer, dissolved by boiling and allowed to gelate. The concentration of the agar is adjusted to 1% that allows amoebae to move outside the boundaries of the drop to increasing concentration gradients of acrasin after cells have degraded chemoattractant in their vicinity by the activity of acrasinases (Konijn 1972). The chemoattractants are added to the agar before pouring plates.

In the modified small population assay (Shimomura *et al.* 1982), small drops of aggregation-competent amoebae are deposited on a hydrophobic agar surface incorporating acrasin of choice, such that all cells remain within the boundary of the drop. Since the developing amoebae secrete acrasinase in their instant vicinity, any acrasin in that region is removed. If there were initially an even concentration of acrasin in the agar, there will now be much less or none within and surrounding the drop of amoebae,

resulting in an increasing concentration gradient from the centre of drop outwards. This gradient causes the orientation of the amoebae beyond the margins of the drop displaying chemotactic sensitivity of cells to the acrasin. An optimal chemotaxis response can be obtained in this assay only when cells are at the right stage of aggregation competence that lasts only for a very restricted period of time; once starving cells cross this short aggregation-sensitivity peak, they move back to the new centres that have developed within the drop and start to secrete their own acrasin (Shimomura *et al.* 1982). Direction of movement of cells is reversed to the centres formed in the original drop because acrasin gradient is reversed (Futrelle *et al.* 1982). The success of the bioassay, then, is dependent on the ability of cells to detect extracellular acrasin and release a stable extracellular enzyme 'acrasinase' which destroys the attractant; properties that are essential for chemotaxis during the normal development of the social amoebae.

To perform chemotaxis assays in this study, the revised small population assay (Shimomura *et al.* 1982) was adapted. To induce aggregation competence, cells were starved for 3-4 hours in shaken suspensions as suggested by Browning and colleagues (Browning *et al.* 1995). This treatment is reported to trigger a spectrum of molecular and biochemical changes that result in the loss of sensitivity to vegetative-stage folate, while inducing responsiveness to aggregation-specific chemoattractant (Browning *et al.* 1995). This assay was first verified by investigating the chemotactic activity of *P. violaceum* to its known acrasins i.e. glorin (Figure 7). Source of glorin used in this assay was the commercial glorin. Different concentrations of glorin were tested (data not shown) and optimal chemotactic response was detected with 1 µM glorin (final concentration; Figure 7).

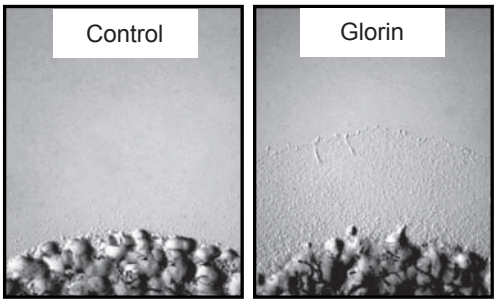


Figure 7: Validation of chemotaxis assay. *P. violaceum* cells developing in the absence (left picture) or presence of 1 µM of glorin final concentration (right pictures). In each experiment, cells were starved in slow shaking buffer suspensions for 4 hrs prior to plating as 10 µl drops containing $2x10^5$ cells on hydrophobic agar. In control plates containing no acrasin, cells remained confined inside the boundaries of the drop. If acrasin was embedded in the agar, amoebae crossed the

margins of the drop in all directions and drop was completely dispersed. Edges of drops were photographed 3 hours after plating to document outward movement of cells from the boundaries of drop.

3.2 Chemotactic specificity of group 4 species

Acrasin of the well-known group 4 species *D. discoideum* is known to be cAMP. Chemotaxis assay adapted in this study was further validated by evaluating response of *D. discoideum* NC4 to cAMP (Figure 8). Different concentrations of cAMP were tested (data not shown) and optimal chemotactic response was noticed with 100 µM final concentration of cAMP (Figure 8).

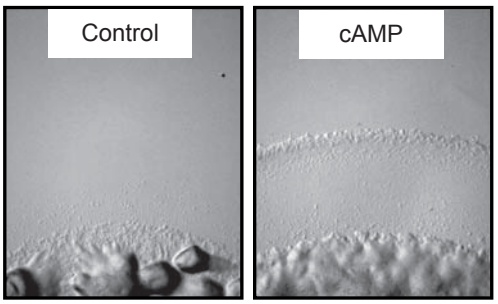


Figure 8: Chemotaxis assay for cAMP used in this study. *D. discoideum* NC4 cells were deposited on hydrophobic agar plates containing either no cAMP (left picture) or 100 µM of cAMP (right picture). In each experiment, cells were starved in slow shaking buffer suspensions for 4 hrs prior to plating as 10 µl drops containing $2x10^6$ cells on hydrophobic agar. In control plates containing no acrasin, cells remained confined inside the boundaries of the drop. If acrasin was embedded in the agar, amoebae crossed the margins of the drop in all directions and drop was completely dispersed. Edges of drops were photographed 3 hours after plating to document outward movement of cells from the boundaries of drop.

Under similar conditions, response of five other group 4 species was evaluated towards cAMP and glorin. All tested group 4 species: *D. sphaerocephalum, D. giganteum, D. dimigraforum, D. firmibasis and D. intermedium* reacted well to cAMP but showed no response to glorin. These data are summarized in Table 1.

3.3 Chemotactic specificity of *P. violaceum* lying at the edge of group 4

The violet coloured *P. violaceum* is distinctly placed at the base of group 4 or in group 3 plus 4 (Figure 1; Schaap *et al.* 2006). Group 4 species use cAMP as chemotactic aggregant, whereas in *P. violaceum*, dipeptide glorin mediates aggregation. Chemotaxis

of *P. violaceum* amoebae to glorin was reported by some groups (Shimomura *et al.* 1982; Wurster *et al.* 1976) and verified in this study (Figure 7 & 9).

3.3.1 Stage specificity in the response of *P. violaceum* amoebae to different chemotactic agents

Previously, it was reported that folic acid causes a strong chemotactic response in the early preaggregation cells of *P. violaceum* (Wurster *et al.* 1978). This study showed that post-vegetative cells starved for 1 hour in shaken suspensions were sufficiently active to cAMP (Figure 9). Sensitivity to cAMP decreased with increase in starvation as shown by a minimal response to 100 µM cAMP by *P. violaceum* amoebae starved for 3 hours.

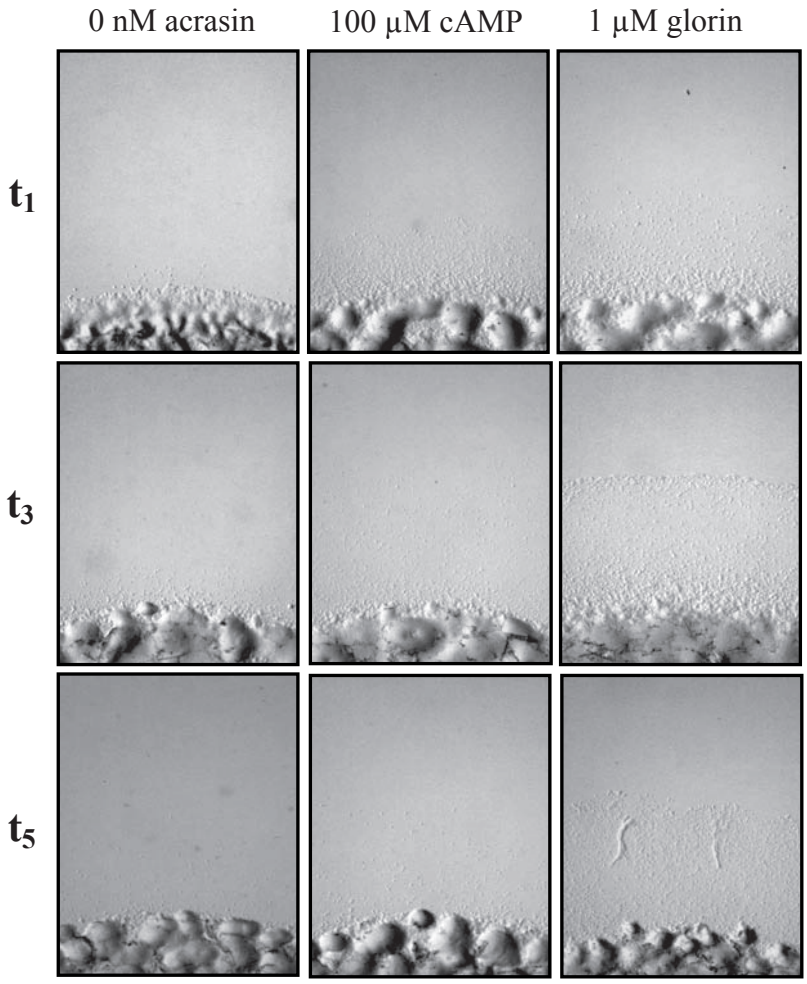


Figure 9: Chemotactic activity of starving *P. violaceum* amoebae to cAMP and glorin. Cells were starved for 1, 3 and 5 hours (t_1, t_3, t_5) in shaking cultures before 10 µl drops containing $2x10^6$ cells were placed on phosphate agar plates prepared with either 100 µM cAMP or 1 µM final

concentration of glorin. Plates containing 0 nM acrasin represented “controls without acrasin”. Chemotaxis towards glorin was monitored every 30 minutes using an Olympus steromicroscope and pictures were taken 3 hours after plating.

Cells starved for 1 hour were adequately active to glorin, while aggregation competence induced by starving cells for 3 or 5 hours in suspensions provoked cells to display greater chemotaxis towards glorin. When cells were plated from cultures starved for 5 hours, a maximal response to glorin was observed where *P. violaceum* amoebae moved out of drop in a dense front with some streams. These results are in agreement with the observations made by Jones and Robertson (1976), which showed that *P. violaceum* amoebae are weakly attracted to a microelectrode releasing 100 µM or 1 mM cAMP (Jones and Robertson 1976). Owing to the phylogenetic position of *P. violaceum* at the bottom of group 4, the weak chemotactic response of starving amoebae of this species to cAMP may represent development of initial biochemical steps towards switching on the use of cAMP to coordinate aggregation that is characteristic of group 4 species.

3.4 Chemotaxis of social amoebae towards glorin is an ancient response

3.4.1 Chemotactic response of group 2 species to glorin

3.4.1.1 Chemotactic specificity of *P. pallidum* PN500 cells

Previously, *P. pallidum* amoebae have been described to be chemotactically reactive to glorin (Shimomura *et al.* 1982; Wurster *et al.* 1976). A group 2 species designated as *P. pallidum* PN500 is emerging as an eminent eukaryotic model system to study fundamental problems in cell and developmental biology because it is accessible to various genetic and biochemical approaches, and imaging techniques (Schaap 2011a). Additionally, recent availability of the genome sequence of *P. pallidum* PN500 (Heidel *et al.* 2011) has opened exceptional possibilities to identify developmentally relevant genes. The ultimate objective of this project was to examine glorin-mediated regulation of gene expression employing *P. pallidum* PN500 as test species. Therefore, as a first step, chemotactic response of *P. pallidum* PN500 towards glorin was investigated. This study demonstrated that *P. pallidum* PN500 amoebae exhibit marked chemotactic orientation and directional locomotion of cells towards glorin but no response was noticed towards cAMP (Figure 10).

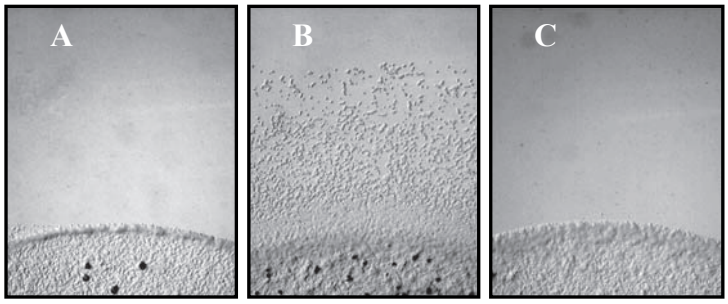


Figure 10: Chemotactic response to glorin by *P. pallidum* PN500. Cells were starved for 3 hours in shaking cultures before 10 µl drops containing $2x10^6$ cells were spotted on phosphate agar plates prepared without (picture **A**), with 1 µM final concentration of glorin (picture **B**) or with 100 µM cAMP (picture **C**). Chemotaxis towards glorin was monitored every 30 minutes using an Olympus steromicroscope and pictures were taken 3 hours after plating. *P. violaceum* was used as a positive control (data not shown).

These data provide an indication that developing *P. pallidum* PN500 amoebae are capable to detect glorin, degrade it and move up a spatial gradient of chemoattractant demonstrating that glorin may be used as acrasin by *P. pallidum* PN500 to coordinate aggregation; therefore, it could be interesting to examine further cellular response to extracellular glorin using *P. pallidum* PN500 as model species.

3.4.1.2 Determination of optimal concentration of glorin required to obtain maximum chemotactic response of *P. pallidum* amoebae

In order to determine optimal concentrations of glorin required for the radial chemotaxis assays, *P. pallidum* PN500 'vegetative stage cells' and 'cells starved for 3 hours' were deposited in form of drops on thin agar plates containing a range of concentrations of glorin. Depending upon the chemoattractant concentration used, cells responded to varying degrees by moving out of the drop to a certain distance. Patterns of response were recorded approximately 3 hours after plating (Figure 11). In these experiments, *P. pallidum* aggregation-competent amoebae migrated '*as individuals'* on plates containing 10-100 nM glorin, while cells migrated outward '*as aggregates'* in response to 1 µM glorin (Figure 11). These results indicate that 100 nM (final concentration) is the optimal concentration of glorin required to observe maximal chemotaxis response of *P. pallidum* PN500 cells (Figure 11). Nevertheless, prominent chemotactic response was detected using even higher concentration of glorin, i.e. 1 µM (Figure 11).

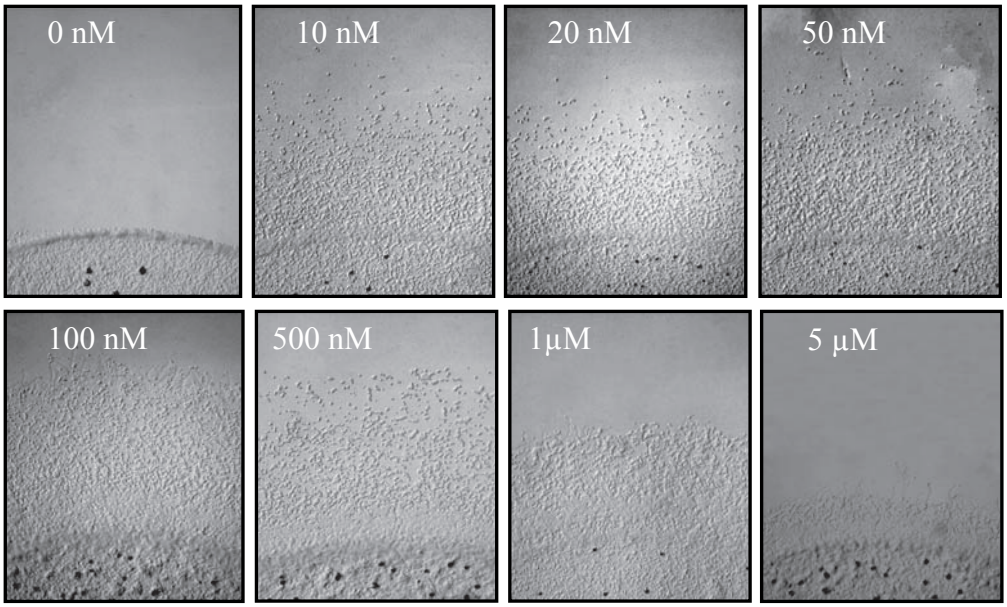


Figure 11: Chemotactic response of *P. pallidum* PN500 amoebae to differing concentrations of glorin. Cells were starved for 3 hours in shaking cultures before 10 µl drops containing $2x10^6$ cells were placed on phosphate agar plates prepared with 0 nM, 10 nM, 20 nM, 50 nM, 100 nM, 500 nM, 1 µM and 5 µM final concentration of glorin. Chemotaxis towards glorin was monitored every 30 minutes using an Olympus steromicroscope and pictures were taken 3 hours after plating.

3.4.1.3 Stage specificity in the response of *P. pallidum* PN500 cells to glorin

These experiments were designed to trace changes in the sensitivity of the amoebae as they "age" in shaking cultures. Vegetative stage *P. pallidum* PN500 cells (indicated by $\mathbf{t_0}$; Figure 12) deposited on hydrophobic phosphate agar plates showed a very weak response to 10 nM glorin and were insensitive to higher concentrations of acrasin (Figure 12). Effects of varying starvation times (from 0- to 4-hours) on the ability of cells to respond to glorin were then assessed. Amoebae starved for 1 hour (indicated by $\mathbf{t_1}$) were moderately more reactive to low concentrations of glorin (Figure 12). The progression of starvation led to an enhanced sensitivity of the responding amoebae, and as a result orientation of *P. pallidum* PN500 cells to the gradients of glorin increased. Cells starved for 2 hrs (data not shown) were adequately active to glorin, while there was a sudden rise in the sensitivity of cells starting at about 3 hours of starvation (indicated by $\mathbf{t_3}$; Figure 12). It was observed that cells developed for 3 hours in shaking suspensions were at the appropriate stage of aggregation competence and responded maximally on chemotaxis assay plates containing optimal concentration of glorin (i.e. 100nM glorin as shown in Figure 11 & Figure 12). When amoebae starved in shaking cultures for 4 hours (indicated

by t_4) were deposited on hydrophobic agar, chemotactic efficiency of the responding cells was comparatively reduced (Figure 12). These data support stage-specificty in the response of *P. pallidum* PN500 cells to glorin.

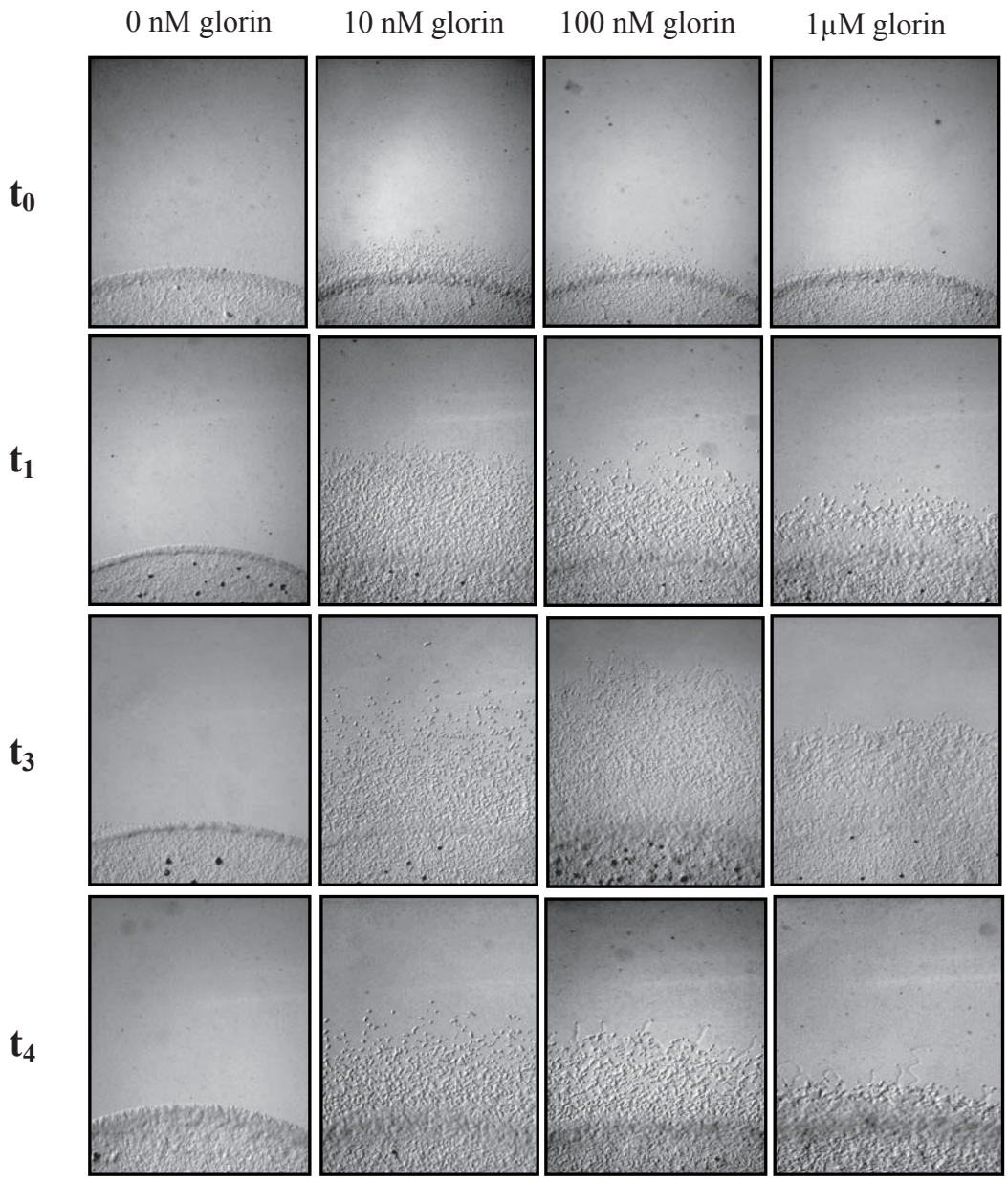


Figure 12: Effects of varying starvation times on the ability of *P. pallidum* PN500 amoebae to respond to glorin. Cells were starved for 1-4 hours (t_1, t_2, t_3, t_4) in shaking cultures before 10 µl drops containing $2x10^6$ cells were placed on phosphate agar plates prepared with 0 nM, 10 nM, 100 nM and 1 µM final concentration of glorin. Chemotaxis towards glorin was monitored every 30 minutes using an Olympus steromicroscope and pictures were taken 3 hours after plating.

3.4.1.4 Delayed chemotactic response of *P. pallidum* amoebae to high concentrations of glorin

When sensitivity of aggregation-competent *P. pallidum* PN500 cells was tested to higher concentrations of glorin, i.e. 10-20 µM glorin, it was noticed that starving amoebae could sense a higher concentration of attractant, however, they responded by relatively slow outward migration from the margins of the drop and did not move a greater distance away from the drop during the first few hours (indicated by $\mathbf{t_1}$ & $\mathbf{t_3}$ in Figure 13) after being plated on hydrophobic agar surface.

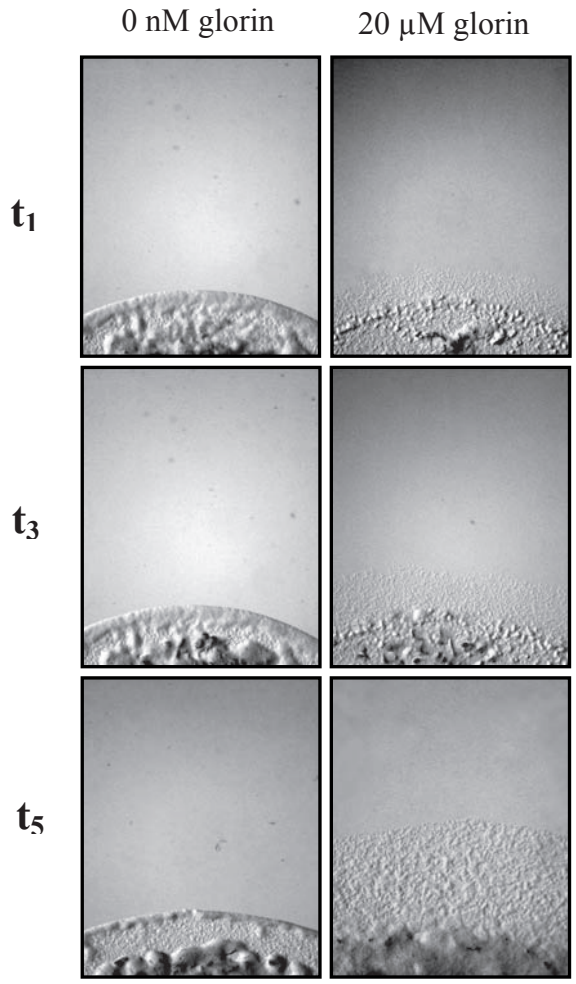


Figure 13: Chemotactic response of starving *P. pallidum* PN500 amoebae to high concentrations of glorin is delayed. Cells were starved for 3 hours in shaking cultures before 10 µl drops containing $2x10^6$ cells were placed on phosphate agar plates prepared with 0 nM or 20 µM final concentration of glorin. Chemotaxis towards glorin was monitored every 30 minutes using an Olympus steromicroscope and pictures were taken at 1, 3 and 5 hours (t_1, t_3, t_5) after plating. Slow migration of amoebae outside the borders of the drop was seen within 1 hour of placing the drop on agar surface, later this outward movement ceased considerably and the

optimal response was recorded at 5 hour of plating. Hence chemotaxis to excess of glorin was delayed by a few hours. Interestingly, in response to higher glorin concentrations, amoebae moved out in a dense front. Similar observations were made when glorin was tested at a concentration of 10 µM.

It seemed clear that the chemotactic response of cells to the excess of glorin was delayed by 2-3 hours (compare results depicted in Figure 12 and Figure 13 at $\mathbf{t_3}$ for cells starved for 3 hours before being deposited on agar). As shown in Figure 12, cells starved in shaking suspensions for 3 hours exhibited significant chemotactic response to 'lower' concentrations of attractant when plated on agar plates and amoebae could travel a considerable distance away from the boundaries of drop (Figure 12). Similar observations were made by De Wit & Konijn (1983), which showed that the chemotactic response of *D. minutum* to high concentrations of folic acid was delayed (De Wit & Konijn, 1983). This phenomenon may indicate that *Dictyostelids* exhibit adaptation during chemotactic migration, undergoing consecutive phases of desensitization and resensitization in the presence of increasing concentrations of acrasins. Such adaptive behaviour allows the social amoebae to reversibly adjust their sensitivities over a wide range of concentrations of the chemoattractant; an essential feature for long-range chemotaxis.

3.4.1.5 *P. pallidum* is a species complex

P. pallidum has been considered as a 'species complex' (Romeralo *et al.* 2011b; Romeralo *et al.* 2010; Kawakami & Hagiwara 2008; Schaap *et al.* 2006; Raper 1984) containing several morphologically similar species that are characterized by white sorocarps carrying elliptical spores and bearing sorophores without lengthened terminal segments (Hagiwara 1989, 1982). This species complex encompasses *Polysphondylium pallidum* Olive (Olive 1901) and its related species *Polysphondylium album* Olive (Olive 1901). *P. album* Olive is distinguished from *P. pallidum* primarily in having larger sori, prostrate sorocarps and larger number of branches per whorl (Kawakami & Hagiwara, 2008). A recent morphological reconsideration of *P. pallidum* species complex by Kawakami and Hagiwara (2008) proposed that *P. pallidum* isolate PN500 (Figure 14) should be renamed as *P. album,* that was placed by Raper together with *P. pallidum* (Kawakami & Hagiwara 2008; Raper 1984). *P. pallidum* isolate PN500 (Figure 14) is characterized by round to clavate sorophore bases, typically ovoid and shorter tip cells and exhibit higher whorl index values (Kawakami & Hagiwara, 2008). However, the isolate *P. pallidum* CK8 (Figure 14) is reported to have lower whorl index values, typically subulate and longer tip cells and clavate sorophore bases (Kawakami and Hagiwara,

2008). Other isolates, such as *P. pallidum* H168 (reported by Romeralo *et al.* 2010) and *P. pallidum* WS320 (Figure 14) are clearly demarcated by a combination of the shape of sorophore base, the number of branches per whorl, and the shape and length of tip cell. In the presented research work, ITS DNA sequencing (Romeralo *et al.* 2010) was used to show that *P. pallidum* strain WS320 is closely related to the isolate PN500 (placed in subclade 2B1; Romeralo *et al.* 2010) and both may therefore be designated as *P. album* isolates (Figure 15). It is further showed that *P. pallidum* CK8 (Kawakami & Hagiwara 2008) is closely related to H168 isolate (placed in subclade 2B2; Romeralo et al. 2010), suggesting that they should be recognised as *P. pallidum* sensu strictu (Figure 15). When compared, the ITS sequences of isolates WS320/PN500 (group 2B1) and CK8/H168 (group 2B2) revealed low conservation, except in the 5.8 rRNA gene sequence, manifesting the relative phylogenetic distance between different isolates of the *P. pallidum* species complex (Figure 15; approved by Romeralo M. and Baldauf S.). It is not yet approved by the community that isolates, such as PN500 or WS320 will be renamed *P. album*, therefore, these strains are ascribed as *P. pallidum* in this study.

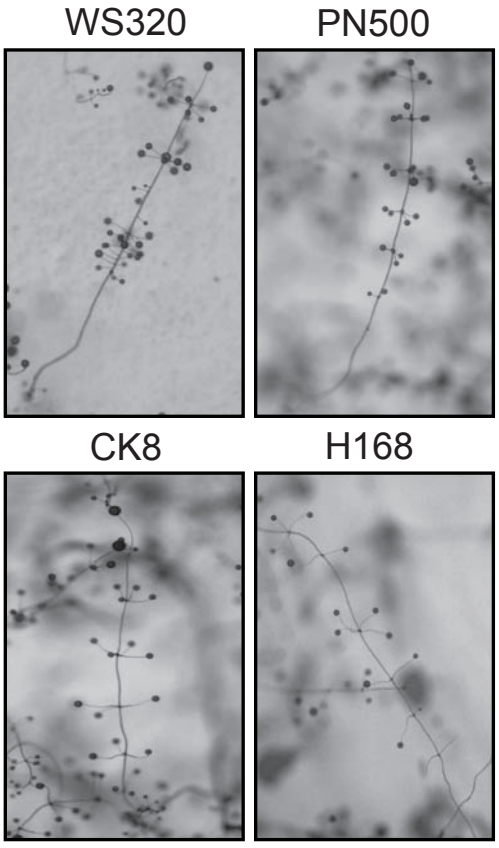


Figure 14: Fruiting body morphologies of different isolates of *P. pallidum*. Fruiting bodies are adorned with regular whorls of side branches. *P. pallidum* WS320 (upper left); *P. pallidum* PN500 (upper right); *P. pallidum* CK8 (lower left); *P. pallidum* H168 (lower right).

```
PN500     TAAAAAAACGAACTCAAAAGGTGTATTAAGAAGCTTCGGTTTCATTTTACACCGCTCTTT
WS320     TAAAAAA-CGAACTCAAAAGGTGTATTAAGAAGCTTCGGTTTCATTTTACACCGCTCTTT
CK8       CATAAAAAAGAACTCAAAAAAAAAAAGTTATTTATTTAACTTTTGCCTAATTT---TTTTA
H168      CATAAAAAAGAACTCAAAAAAAAAAAGTTATTTATTTAACTTTTGCCTAATTT---TTTTA
             ***** **********     * * *     **   ***    * *      * **

PN500     GCTAAAAATTCTTCGGAATTTAAGCTTA---GAGTATCTCAAAAAA--ACTTATTTCGTA
WS320     GCTAAAAATTCTTCGGAATTTAAGCTTA---GAGTATCTCAAAAAAAAACTTATTTCGTA
CK8       ATTTGGTATTCATATTAAATTAAATTAATTTGGGCATCTCTCTCAAA-ATTTATTTCGTA
H168      ATTTGGTATTCATATTAAATTAAATTAATTTGGGCATCTCTCTCAAA-ATTTATTTCGTA
            *    **** *   ** ****  * *   * * *****    **  * **********

PN500     TGTCAAATTCAATGTAGTTGTCTGATAGTCTAATGACATCCTCGGATGTTCCAATTCTTG
WS320     TGTCAAATTCAATGTAGTTGTCTGATAGTCTAATGACATCTTCGGATGTTCCAATTCTTG
CK8       TGTCAAATTAATTGTAATTGTCTAA---------GATGTTTCCACTCTGTGGAAATATTA
H168      TGTCAAATTAATTGTAATTGTCTAA---------GATGTTTCCACTCTGTGGAAATATTA
          ********* * **** ****** *          **  *   *      *  ** * **

PN500     AAGTCAATGATTTTTAGTAAGCATAAATGGTGGATACCTCGGCTCCTAAATCGATGAAGA
WS320     AAGTCAATGATTTTTAGTAAGCATAAATGGTGGATACCTCGGCTCCTAAATCGATGAAGA
CK8       AAGTCAATGATTTTTAGTAAGCATAAATGGTGGATACCTCGGCTCCTAAATCGATGAAGA
H168      AAGTCAATGATTTTTAGTAAGCATAAATGGTGGATACCTCGGCTCCTAAATCGATGAAGA
          ************************************************************

PN500     CCGTAGCAAACTGCGATAAGTCACTTGAATTGCAGACTACTGTGAAAGTCGAACTGTTGA
WS320     CCGTAGCAAACTGCGATAAGTCACTTGAATTGCAGACTACTGTGAAAGTCGAACTGTTGA
CK8       CCGTAGCAAACTGCGATAAGTCACTCGAATTGCAAACTACTGTGAAAGTCGAAATGTTGA
H168      CCGTAGCAAACTGCGATAAGTCACTCGAATTGCAGACTACTGTGAAAGTCGAAATGTTGA
          ************************ ******** ****************** ******

PN500     ACGCACATGATGATATTGATTCTTCACGGAGTTAAATATCACACTTGGTTGAGAGTCGCA
WS320     ACGCACATGATGATATTGATTCTTCACGGAGTTAAATATCACACTTGGTTGAGAGTCGCA
CK8       ACGCACATGATGACATTGACTCCCCC--GAGTTAAATGTCACACTTGGTTGAGAGTCGCA
H168      ACGCACATGATGACATTGACTCCCCC--GAGTTAAATGTCACACTTGGTTGAGAGTCGCA
          ************* ***** **  *   ********* ***********************

PN500     TCTCATTATCATTTACCAATAGTTTTTTTTTCGAAAAAGCTATTGGCAAAGTATTGGGAA
WS320     TCTCATTATCATTTACCAATAGTTTTTTTT-CGAAAAAGCTATTGGCAAAGTATTGGGAA
CK8       TCTCATTATCATTTCTTAATTCTATT-------AAGAAAGTATTGGTAAAGATTTCTTAG
H168      TCTCATTATCATTTCTTAATTCTATT-------AAGAAAGTATTGGTAAAGATTTCTTAG
          **************   ***  * **       ** **  ****** ****  **   *

PN500     AGTTTTCTATGACAATTCGGGATTACTTTCTTTAAATATTAAAACATTGTCAGGTATGAA
WS320     AGTTTTCTATGACAATTCGGGATTACTTTCTTTAAATATTAAAACATTGTCAGGTATGAA
CK8       A------TATTACA-TTCGGAAT--CTTCACTTAAATATAAAAAAAAAAAAGTAATATCAG
H168      A------TATTACA-TTCGGAAT--CTTCACTTAAATATAAAAAAAAAAAAGTAATATCAG
          *      *** *** ***** **  ***   ******** **** *        *** *

PN500     AATTAGTTGTCTCACGACTTCTAATATCCAATAGATTCAAAATTATTTAATTGGTTTCCA
WS320     AATTAGTTGTCTCACGACTTCTAATATCCAATAGATTCAAAATTATTTAATTGGTTTCCA
CK8       GTACAATTCCAATAAGAAACAAAATCGTTTTCCAATTTGT--TTCCGCAAGGAAACAGCA
H168      GTACAATTCCAATAAGAAACAAAATCGTTTTCCAATTTGT--TTCCGCAAGGAAACAGCA
              * **     * **     ***          ***     **    **        **

PN500     GTTTACTGGAGTGGCCAGTTGGATTTTTTAAAGTCTTTGTTCGTTTCGACTAAATTTTTA
WS320     GTTTACTGGAGTGGCCAGTTGGATTTTTTAAAGTCTTTGTTCGTTTCGACTAAATTTTTA
CK8       ATTGAATAATTTTTTTGTTATAAATTTTTAACAA--TTATTGATTTCGATTCAATTTTT-
H168      ATTGAATAATTTTTTTGTTATAAATTTTTAACAA--TTATTGATTTCGATTCAATTTTT-
           ** * *    *       *   * *******     ** **  ****** * *******

PN500     GTGGAACGGGATTGGACTGAAGCATTTGCTTCTTTTTCATATAAAAACTTTAATTGGTTA
WS320     GTGGAACGGGATTGGACTGAAGCATTTGCTTCTTTTTCATATAAAAGCTTTAATTGGTTA
CK8       GTGAGAAAATTTCGGATAGTACGAAGTTTTGATCGTTAAT-TCAAGGATGGGATTTACAA
H168      GTGAGAAAATTTCGGATAGTACGAAGTTTTGATCGTTAAT-TCAAGGATGGGATTTACAA
          ***  *     * ***  * *  *  *  *  *  ** ** * **   *   ***    *

PN500     AACCCAGTAACTATAGTTGATAAATCGACTTAGCTATTAATGAAAGTTTTAAACATCAAA
WS320     AACCCAGTAACTATAGTTGATAAATCGACTTAGCTATTAATGAAAGTTTTAAACATCAAA
CK8       AATCTTA-AACTGGTAATGAAAAATTAA---AACTATCAAT------TTGAAAGATTGCC
H168      AATCTTA-AACTGGTAATGAAAAATTAA---AACTATCAAT------TTGAAAGATTGCC
          ** *    ****     *** ****  *   * **** ***      ** *** **

PN500     TTGAAAAGTCTAACTTTCCAACGTTTAGAATTAGTAGTTTCTGACGCGTCTTTTGTGTAG
WS320     TTGAAAAGTCTAACTTTCCAACGTTTAGAATTAGTAGTTTCTGACGCGTCTTTTGTGTAG
CK8       TCGCGTGATTT---TTTAAAATGTTTTAAATTAGTAATTATCG-TATGTTTTT------G
```

```
H168      TCGCGTGATTT---TTTAAAATGTTTTAAATTAGTAATTATCG-TATGTTTTT------G
          * *     * *   ***  ** ****  ******** **   *    ** ***      *

PN500     ACTATAGCAGAAAAGTTTAACGCTAACAATTTTTGTTTTTGAAAAAGTAGTAGTCTAGTA
WS320     ACTATAGCAGAAAAGTTTAACGCTAACAATTTTTGTTTTTGAAAAAGTAGTAGTCTAGTA
CK8       ATTATTGTAGACTTGCACTGCTTTAACA-----CATGGTTAAAATTTTGGAAATT---TG
H168      ATTATTGTAGACTTGCACTGCTTTAACA-----CATGGTTAAAATTTTGGAAATT---TG
          * *** * ***   *     *  *****       *  ** ***   * * * *    *

PN500     AGTACTTTCTCTAGTAAACTTTAAGTTTTTTAACGCTTCGAGCAGCTACAAGACTTTTTA
WS320     AGTACTTTCTCTAGTAAACTTTAAGTTTTTTAACGCTTCGAGCAGCTACAAGACTTTTTA
CK8       AGAATTTTTCATATTAATTGAAATGATTCCGGTAATGACATAAAATTTTTAGCATATATA
H168      AGAATTTTTCATATTAATTGAAATGATTCCGGTAATGACATAAAATTTTTAGCATATATA
          ** * ***   ** ***     * * **          *    *  *   **  * * **

PN500     AGATTCAATTTGACTATCAATTATGGCTATTAAGTAATTTTGTATACCTTCTCAAAAATC
WS320     AGATTCAATTTGACTATCAATTATGGCTATTAAGTAATTTTGTATACCTTCTCAAAAATC
CK8       AAAGTTA-TCCAACTGTCAATTATGGTTGTTAAATTTTTTTTTTGAGTACCTACAAAAATT
H168      AAAGTTA-TCCAACTGTCAATTATGGTTGTTAAATTTTTTTTTTGAGTACCTACAAAAATT
          * * * * *   *** ********** * **** *  **** *         *******

PN500     TTTGGACTT----TTAAAAGTTGAATTGA-CGCTGT-TTGTTCACTCAATTTAACTATAA
WS320     TTTGGACTT----TTAAAAGTTGAATTGA-CGCTGT-TTGTTCACTCAATTTAACTATAA
CK8       CTTTGGTTCCTTATTAACAAATGTTTTAAACACTGTATTGTTAAGTTTTTAACATTTTAA
H168      CTTTGGTTCCTTATTAACAAATGTTTTAAACACTGTATTGTTAAGTTTTTAACATTTTAA
           ** *  *     **** *  **  ** * * **** ***** * *   *   * * ***

PN500     AA-AAAC-CTACAAAAAGCGACATCAG
WS320     AA-AAAC-CTACAAAAAGCGACATCAG
CK8       AATAAACACTTCCAAAAGCGACATCAG
H168      AATAAACACTTCCAAAAGCGACATCAG
          ** **** ** * **************
```

Figure 15: Alignment of ITS sequences from *P. pallidum* isolates, namely, PN500, WS320, CK8, and H168 generated with CLUSTAL 2.0. Nucleotide positions that are identical in all four sequences are indicated by stars. The 5.8 rRNA gene is illustrated in blue letters.

3.4.1.6 Chemotactic response of different *P. pallidum* isolates and group 2 Dictyostelid species to glorin

Next chemotactic response of different *P. pallidum* isolates, such as WS320, CK8 and H168 to glorin was tested. In these experiments, all *P. pallidum* isolates reacted equitably well to glorin. Eight other group 2 species were assayed for their responsiveness to glorin. The results are summated in Figure 16 and Table 1. It was observed that the group 2B1 polysphondylids *P. pseudocandidum*, *P. asymmetricum*, *P. tenuissimum* and the group 2B2 species *P. tikaliensis* and *P. luridum* were chemotactically active to glorin. Qualitatively, these polysphondylids reacted to glorin as efficiently as *P. pallidum* isolates did. Two *Dictyostelium* species placed in group 2, *D. gloeosporum* and *D. oculare* also responded to glorin equally well (Figure 16), while *Acytostelium* species were not found positive in this assay (Table 1). None of the tested group 2 species responded to cAMP (Table 1). These data indicate that glorin-based cell-cell communication is a common property of early diverged group 2 species.

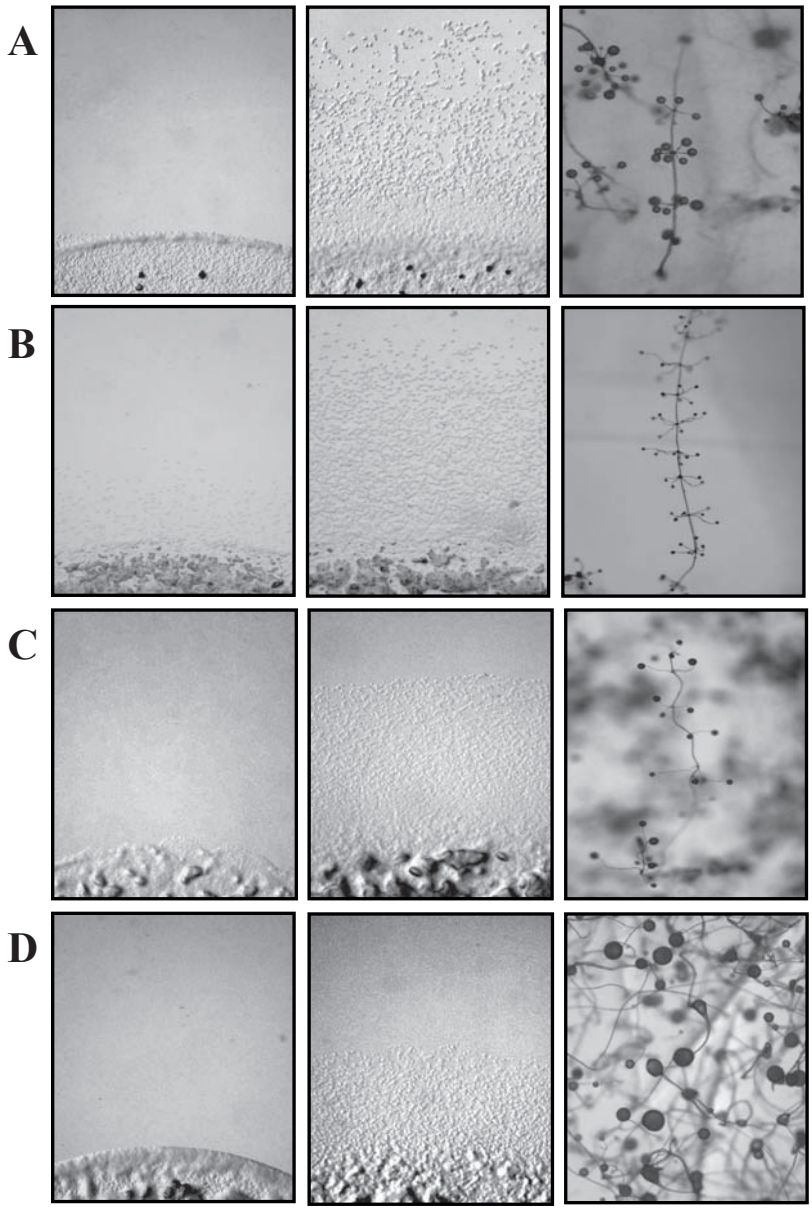


Figure 16: Chemotactic response to glorin by group 2 species. Cells were starved for 3 hours in shaking suspensions before 10 µl drops containing $2x10^5$ cells were placed on hydrophobic agar without (left pictures) or with 1 µM glorin (middle pictures). Shape of fruiting bodies of each species is shown (right pictures). Chemotaxis assay pictures were taken 3 hours after plating. **A**: *P. pallidum* PN500; **B**: *P. tenuissimum*; **C**: *P. asymmetricum*; **D**: *Dictyostelium gloeosporum*. *P. violaceum* was used as positive control (data not shown).

3.4.2 Chemotactic response of group 1 species to glorin

Glorin communication was further explored more deeply into the phylogenetic history of social amoebae. It was found that group 1 species *D. fasciculatum*, *D. parvisporum*, *D. aureo-stipes, D. microsporum* and *D. bifurcatum* exhibited pronounced chemotaxis to glorin (Figure 17). It was convincing data to believe that ancient group 1 species

generally employ glorin as extracellular signalling molecule. Group 1 species were not reactive to cAMP in these chemotaxis assays (Table 1).

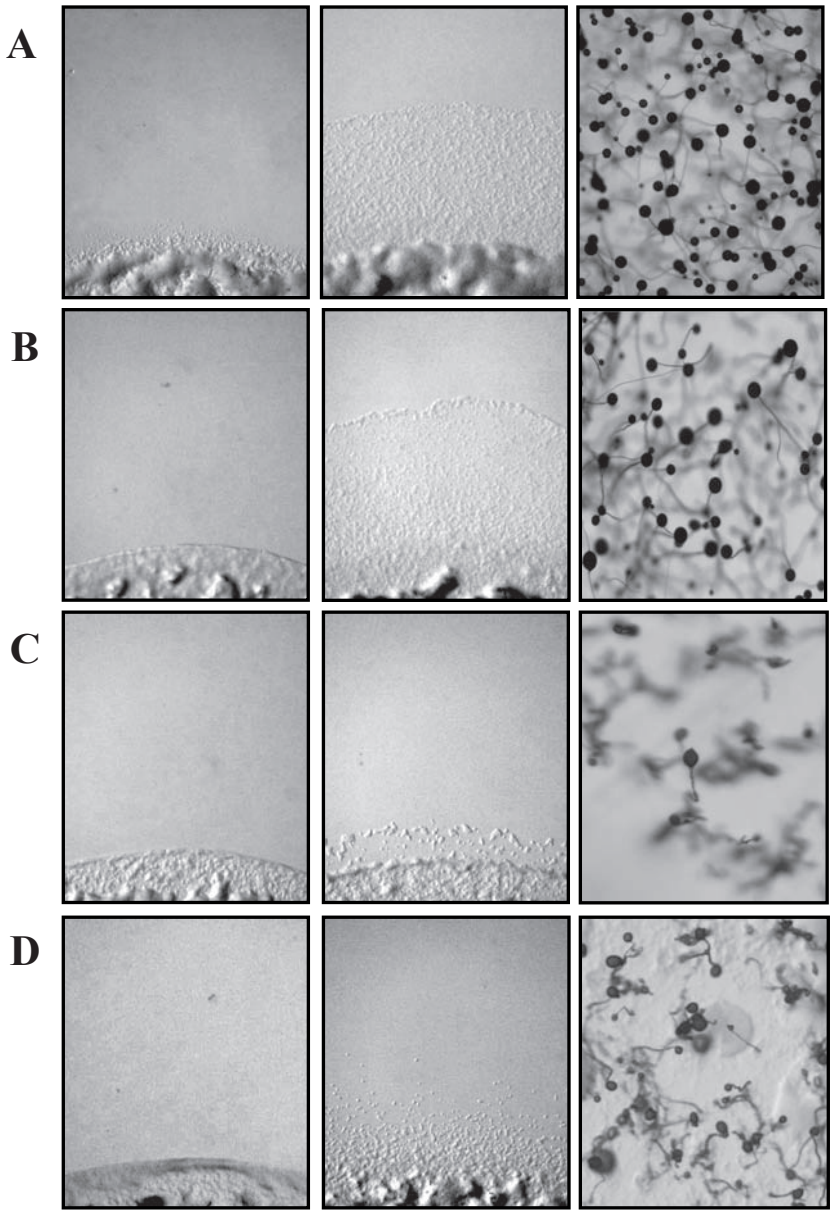


Figure 17: Chemotaxis to glorin by group 1 species. Cells were starved for 3 hours in shaking suspensions before 10 µl drops containing $2x10^5$ cells were deposited on hydrophobic agar prepared without (left pictures) or with 1 µM glorin (middle pictures). Shape of fruiting bodies of each species is shown (right pictures). Chemotaxis assay pictures were taken 3 hours after plating. **A**: *Dictyostelium fasciculatum*; **B**: *D. aureo-stipes*; **C**: *D. parvisporum*; **D**: *D. microsporum*.

Species	Dictyostelids group[a]	Activity to cAMP	Activity to glorin
Dictyostelium discoideum NC4	4	+	–
Dictyostelium discoideum AX2	4	+	–
Dictyostelium sphaerocephalum	4	+	–
Dictyostelium giganteum	4	+	–
Dictyostelium dimigraforum	4	+	–
Dictyostelium firmibasis	4	+	–
Dictyostelium intermedium	4	+	–
Polysphondylium violaceum	(4)	(+)	+
P. pallidum WS320	2B1	–	+
P. pallidum PN500	2B1	–	+
Polysphondylium tenuissimum	2B1	–	+
Polysphondylium asymmetricum	2B1	–	+
Dictyostelium gloeosporum	2B1	–	+
P. pallidum H168	2B2	–	+
P. pallidum CK8	2B2	–	+
Polysphondylium luridum	2B2	–	+
Polysphondylium tikaliensis	2B2	–	+
Dictyostelium oculare	2B	–	+
Acytostelium ellipticum	2B	–	–
Acytostelium subglobosum	2A	–	–
Dictyostelium fasciculatum	1	–	+
Dictyostelium aureostipes	1	–	+
Dictyostelium parvisporum	1	–	+
Dictyostelium microsporum	1	–	+
Dictyostelium bifurcatum	1	–	+

Table 1: Chemotactic activity of aggregation competent amoebae from four groups of Dictyostelids was tested towards glorin and cAMP. Chemotaxis tests were carried out twice for each species. "+" indicates positive chemotactic response. "–"stands for no response.
[a] Group description based on Schaap *et al.* (2006) and Romeralo *et al.* (2010).

In short, these data demonstrate that glorin-based cell-to-cell signalling is the oldest form of intercellular communication used at the transition from growth to multicellular development of social amoebae.

3.5 Identification of the proteins secreted by aggregating *P. pallidum* amoebae: Fishing for putative glorinase

If glorin is the acrasin *of P. pallidum* PN500, then a glorin degrading enzyme must also be secreted in the medium during aggregation of cells to keep extracellular levels of glorin within bounds. In related species *D. discoideum*, during incubation of amoebae under buffered, a rise in the amount of extracellular cyclic AMP phosphodiesterase has been observed, which reaches a peak at the time of aggregation and then declines (Noce *et al.* 1983; Malkinson & Ashworth 1973; Riedel *et al.* 1973; Chassy 1972; Gerisch *et al.* 1972; Chang 1968). Based on these previous reports, it was interesting to search for a glorin degrading enzyme in the medium conditioned by aggregating *P. pallidum* PN500 amoebae to get further insight into glorin communication system. Studies with *P. violaceum* have demonstrated the occurance of extracellular and membrane-bound enzyme(s) capable of inactivating glorin (De Wit *et al.* 1988; Wurster *et al.* 1976). It has been reported that inactivation of glorin signal occurs mainly by rapid cleavage of the δ-lactam bond, followed by a slower cleavage of the peptide bond between propionic acid and glutamic acid (Figure 6; De Wit *et al.* 1988). The same authors showed that considerable degradation of glorin occurs when this dipeptide compound is incubated with extracellular medium of aggregating *P. violaceum* amoebae (De Wit *et al.* 1988). In the presented research work, the 'secretome' of aggregating *P. pallidum* PN500 cells was studied in an attempt to identify a putative hydrolase that would degrade glorin possibly by opening δ-lactam ring.

To collect secreted proteins, vegetatively growing *P. pallidum* PN500 cells were harvested from culture plates using cold 17 mM phosphate buffer (pH 6.2) and freed of bacteria by centrifugation at 800 rpm in phosphate buffer. The final cell pellet was resuspended in phosphate buffer at a concentration of 10^7cells/ml. Cells (10^7) were then pipetted on to Type 353102 1 mm pore-sized polyethylene terephthalate membrane six-well format cell-culture inserts (Figure 18). Within 10 minutes, when the cells had settled on, and attached to the membrane, the buffer was gently removed from the insert. Cold phosphate buffer containing a protease inhibitor cocktail, AEBSF to a final concentration of 0.25mM and 5 mM EDTA was added into the wells of a six-well plate followed by the placement of the inserts with cells in the wells (Figure 18). The volume of phosphate buffer in the wells was adjusted to just touch the membrane of the insert to keep the membrane and the cells moist. Amoebae developed normally at 21℃, with cells aggregation starting at 4 hour and branching streams formation at 8 hours (data not

shown). When amoebae were actively forming aggregation streams on the surface of inserts, the conditioned starvation buffer in the wells was collected in 2 ml eppendorf tubes and stored at -80℃. Frozen conditioned starvation buffer samples were lyophilized and resulting powder in each tube was resuspended in 50 µl of deionized water. Samples were then immediately boiled with 50 µl of 2 x Laemmli buffer for 5 min at 95℃ to denature proteins and subsequently centrifuged for 10 min at 11000 x g to remove the large particles. Supernatant was collected and used for protein analysis. Proteins were then separated on polyacrylamide gel on the basis of size. Silver-staining of the gel revealed the presence of numerous proteins (Figure 19). Protein bands were excised and proteomic analyses of secreted proteins were performed.

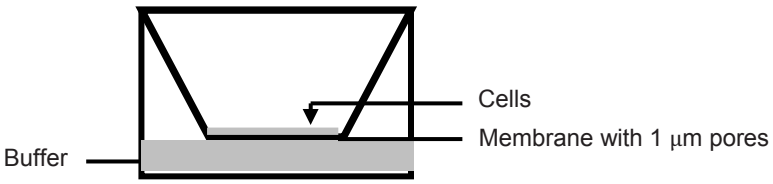


Figure 18: Experimental setup for collection of secreted proteins. *P. pallidum* PN500 cells were placed on a porous membrane in contact with buffer. Amoebae developed normally and aggregation streams could be seen at 8 hours (data not shown). During this period, proteins were secreted into the buffer.

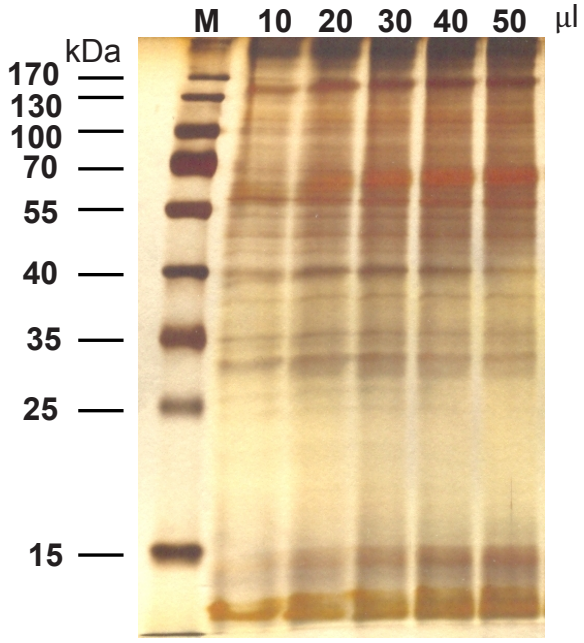


Figure 19: Proteins secreted by aggregating *P. pallidum* PN500 cells. Conditioned buffer was lyophilized and secreted proteins were subjected to separation by electrophoresis on a SDS-

polyacrylamide gel, which was then silver stained. Different volumes of the same protein solution were loaded in each well of 12.5% SDS-PAGE gel to analyze protein band patterns. From left to right, lane1, molecular mass marker (Page Ruler), lane 2, 3, 4, 5, 6 contains 10 µl, 20 µl, 30 µl, 40 µl and 50 µl of concentrated protein solution respectively. A number of protein bands on silver stained SDS-PAGE were rather diffused; this might be because of the heterogeneity of the secreted proteins owing to glycosylation.

A total 97 different proteins were identified including a variety of peptidases and hydrolases. Though, great care was taken to avoid any treatments likely to cause cell lysis, yet presence of some intracellular proteins was identified. The detection of internal proteins in secretome studies due to cell lysis is an inherent aspect of secretome proteomics. A complete list of the proteins identified in two independent experiments is shown in (Appendix Table A1). Same protein species were identified in many different bands (data not shown), revealing that many proteins were digested by proteases during the 8 hours of incubation and sample preparation despite the presence of protease inhibitors.

Most prominent proteins identified by ESI tandem mass spectrometry included members of glycoside hydrolase (GH) families 5, 18, 20, 25, 27, 35 and 39, peptidase families C26, C1A, C53 and S28, gamma-glutamyl hydrolase, glycosyl hydrolase family chitinase, dipeptidyl-peptidase III, beta-N acetylhexosaminidase, putative alpha-N-acetylgalactosaminidase, beta-galactosidase, alpha-glucosidase, beta-xylosidase-like protein, alpha-mannosidase, metallopeptidase, cathepsin Z-like protein and counting factor associated protein. Peptides crorresponding to cathepsin L-like proteinase, discoidin I and acetylornithine deacetylase were also detected. Many of the identified proteins belonged to the category of carbohydrate metabolism. Twenty proteins identified from *P. pallidum* PN500 database were ‘hypothetical proteins’ with unknown functions. For such proteins, homology searches were performed using the BlastP program against all non-redundant protein sequences available at the National Centre for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/blast). However, no putative function could be assigned to these hypothetical proteins. These hypothetical proteins, if unique, may represent good targets for future biochemical studies.

In this study, any putative lactamase could not be detected in the conditioned buffer. It is possible that the lactam hydrolase protein was present, but not in sufficient quantity for detection via applied proteomics, or there is a possibility that it could not be detected as a result of extensive post-translational modifications. After two attempts, more efforts to search for a putative ‘lactam opening hydrolase’ as glorinase candidate or biochemical

characterization of the identified peptidases were not possible because of time limitation. More research therefore needs to be done to identify/define glorinase.

3.6 Genome-wide analysis of glorin modulated gene expression changes

A distinct feature of development of *P. pallidum* is an aggregative transition from a unicellular to a multicellular phase. The outcome of the chemotaxis assays (described under Section 3.4.1.1; Figure 10) using glorin as chemoattractant clearly indicated that *P. pallidum* PN500 cells possess all necessary biochemical machinery required to use glorin as an acrasin. These findings prompted us to address the question whether glorin regulates gene expression changes at the transition from growth to aggregation; similar to the role that chemoattractant cAMP plays in *D. discoideum* during chemotactic aggregation (Winckler *et al.* 2004; Iranfar *et al.* 2003; Mann *et al.* 1997; Schulkes & Schaap 1995; Pitt *et al.* 1993; Mann & Firtel 1987). Previously Will Kopachik (Kopachik 1990) reported effects of glorin on protein synthesis in starving *P. violaceum* amoebae. In his experiments, beginning 1 hour after starvation, *P. violaceum* cells were exposed to glorin (1 µM final concentration, at 30 minute intervals) for 2 to 7 hours. It was demonstrated that within 2 hours of the beginning of starvation, the amoebae displayed two fold increase in sensitivity to glorin. It was found that starving *P. violaceum* cells exhibit ‘some changes’ in protein synthesis when exposed to glorin for only 2 hours, whereas ‘prominent changes’ were observed when cells were treated with glorin for 7 hours (Kopachik 1990). These previous studies further incited us to investigate the putative role of glorin in modulating changes in gene expression patterns of developing *P. pallidum* PN500 cells. To approach this question, we were able to take advantage of recently completed genome sequence of *P. pallidum* PN500 (Heidel *et al.* 2011), while employing Illumina high-throughput RNA sequencing (RNA-seq) technology for analyzing global changes in gene expression.

Glorin-regulated gene expression changes have not been investigated before; therefore, no valuable information was available about optimal conditions to monitor transcriptional changes in response to externally added glorin. Results of the chemotaxis experiments employing *P. pallidum* PN500 as test species (described under Section 3.4.1.3; Figure 12) indicated that amoebae developed in shaking suspension for 1 hour were sufficiently responsive to glorin and gained maximum sensitivity to chemoattractant glorin within next 2-3 hours of development (Figure 12). Also it was noticed that *P. pallidum* amoebae start to aggregate within 3-4 hours of incubation under buffer in petri dishes (Figure 20).

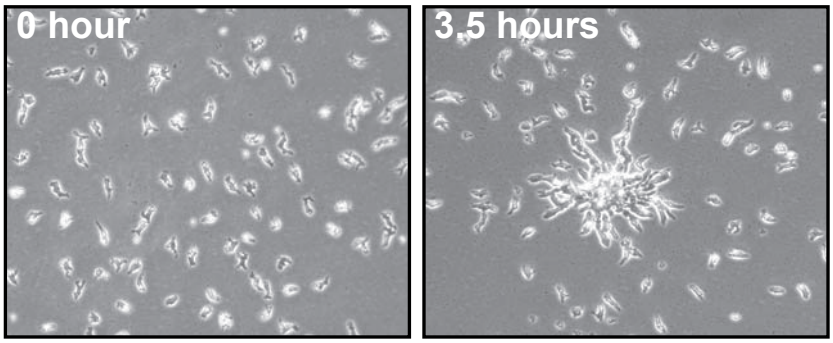


Figure 20: Aggregation of *P. pallidum* PN500 amoebae under buffer. $3x10^7$cells were starved submerged under a thin layer of phosphate buffer in a 50-mm petri dish and monitored for aggregation by differential interference contrast microscopy. Images were taken at 0 hour (left picture) and when cells started to aggregate (right picture).

Previously, it was demonstrated that in starving *P. violaceum* cells glorin acts by binding to cell-surface G-protein coupled receptors (De Wit *et al.* 1988). The same authors reported that *P. violaceum* amoebae developed in shaken suspensions exposed greater number of glorin receptors compared to the cells developed on agar surface (De Wit *et al.* 1988). It was proposed that cells starved in suspension cultures gain maximum sensitivity to glorin because amoebae are not able to aggregate under these conditions and development is arrested in the preaggregation phase (De Wit *et al.* 1988).

Predominant role of G-protein coupled receptors (GPCRs) in *Dictyostelids* is chemoattractant sensing (Heidel *et al.* 2011). GPCRs activate intracellular responses by interacting with heterotrimeric G-proteins. In order to study glorin-induced changes in gene expression patterns of developing *P. pallidum* PN500 amoebae, it was important to identify some genes whose expression was differentially regulated by glorin. However, not many examples of developmentally regulated genes were available for *P. pallidum* PN500. When experiments presented in Section 3.6 of this study were initiated, genome sequence of *P. pallidum* PN500 was not yet published but a list of genes was available to us that may encode G-protein coupled receptor proteins in this species (courtesy of Dr. Gernot Glöckner, Fritz-Lippman Institute Jena, Germany). 25 GPCR genes were randomly selected from the available list (data not shown) and subjected to preliminary analysis of glorin-regulated gene expression.

In this study, initially the protocol devised by Kopachik (Kopachik 1990) was followed to stimulate *P. pallidum* amoebae with glorin. *P. pallidum* PN500 cells grown in association with *Klebsiella planticola* were harvested at vegetatively growing stage, washed free of

bacteria and resuspended in phosphate buffer at cell density $2x10^7$cells/ml. A pellet of $2x10^7$cells was immediately stored at -80^oC and served as 'growing cells control for subsequent gene expression analysis. Cell suspension of *P. pallidum* PN500 amoebae was then divided into two parts. One suspension culture was first prestarved for 1 hour to initiate development, and then treated with periodic additions of 1 µM final concentration of glorin at 30-min intervals for up to 8 additional hours to assess the time-course effects of exogenous glorin on the expression of selected putative GPCR genes. In this experiment, 8 hour time scale was chosen to identify the period when the greatest increase in mRNA levels could be detected. Other culture was maintained in the absence of added glorin for the time period. Suspension cultures (at high cell density $2x10^7$cells/ml) were shaken at low speed i.e. 100 rpm because it may permit cell-cell interactions. Starting at second hour of starvation, cell samples were taken from glorin treated and untreated cultures every hour for total 8 hours to extract total RNA. cDNAs were prepared and expression of chosen putative GPCR genes was analyzed using real-time RT-PCR.

It was taken into account that the final concentration of glorin, i.e. 1 µM applied at 30-minute intervals, used to study glorin-regulated gene expression in suspension cultures of starving *P. pallidum* PN500 cells was higher than the concentration (i.e. 100 nM) found to show optimal response in chemotaxis assays on agar surface for the same cells (Figure 11). This difference could be justified by the fact that for *P. violcauem* cells developing in shaken suspensions, concentration for half maximal occupancy of the major glorin receptor is 200 nM and only 80 nM for cells aggregating on agar surface (De Wit *et al.* 1988). Moreover, it was shown that developing *P. violaceum* amoebae exhibit glorin degradation activity and do not amplify glorin signal in response to externally added glorin; activities that would lead to rapid reduction in initially high concentrations of glorin (De Wit *et al.* 1988). Thus, in this study, final concentration of glorin available to affect gene expression in *P. pallidum* PN500 cells developing in buffer suspensions might be relatively lower and within the range exploited by amoebae for chemotaxis.

Among all tested GPCR genes (data not shown), PPL_04108 was found to be highly induced by glorin at second hour of starvation when cells received glorin treatment for only one hour, i.e. after total 2 additions of glorin, whereas maximal increase in the expression of another GPCR encoding gene, PPL_00855, was noticed at third hour of starvation after stimulation of cells with glorin for total two hours (Figure 21). After these time points, expression of these genes generally declined to lower levels (Figure 21).

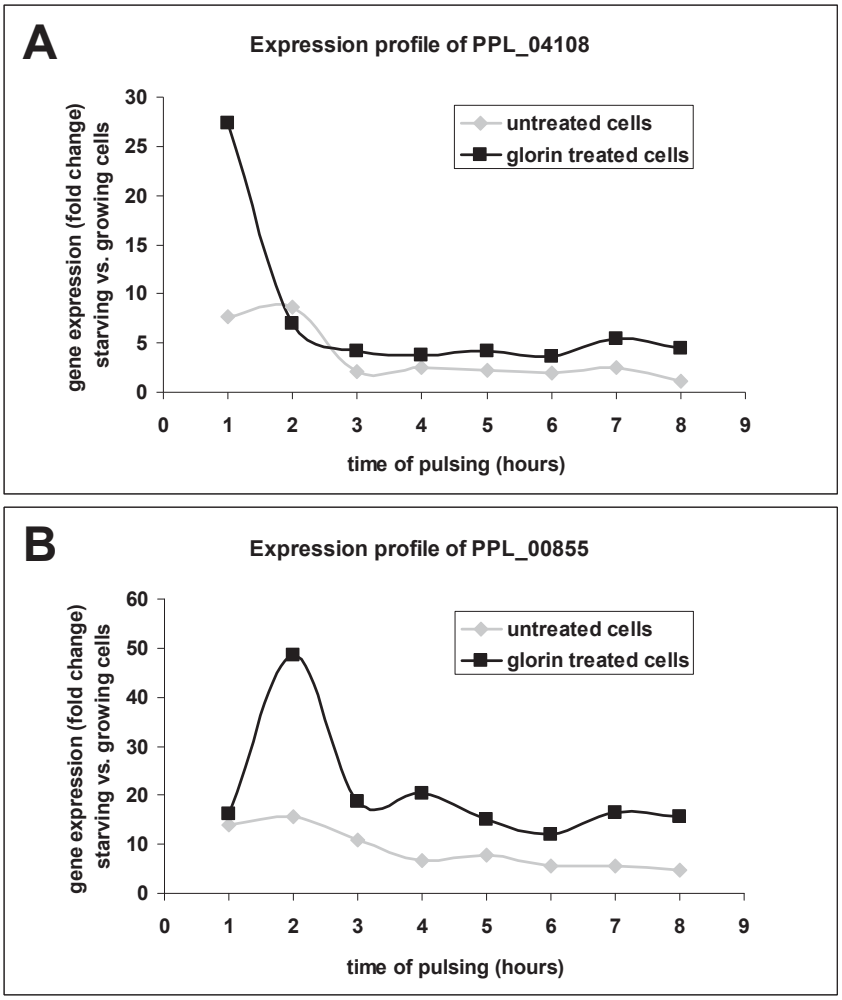


Figure 21: Expression kinetics of two putative GPCR genes during pulse development.
P. pallidum PN500 cells were developed in shaken suspensions and given pulses of 1 µM final concentration of glorin starting at 1 hour of starvation. At the indicated time points, gene expression levels were measured for PPL_04108 and PPL_00855 by quantitative RT-PCR**.** As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represent higher expression of the gene in starving cells than in growing cells. Mean of two independent experiments is presented.

Based on these preliminary data, it seemed interesting to study changes in the patterns of gene expression at 2 and 3 hours after initiation of development because during this time (known as interphase or pre-aggregation stage) cells may undergo many biochemical changes to prepare themselves for imminent aggregation. Therefore, it was decided to perform RNA-seq on cDNAs drived from cells treated with glorin for one and two hours (i.e. cells starved for total two and three hours) to study effects of glorin stimulation on developmentally regulated genes.

For RNA-seq studies, samples of total RNA were prepared from growing cells (**t0**), cells developed for 2 hours without glorin treatment (**t2**), cells developed for 3 hours without glorin treatment (**t3**) and cells pre-starved for 1 hour and then stimulated with 1 µM final concentration of glorin for 1 and 2 hours, respectively (**t2+** and **t3+**) in two independent biological replicates and analyzed by RNA-seq to obtain gene expression profiles from steady-state mRNAs (data deposited in the Gene Expression Omnibus (GEO) database under accession number GSE24911). In short, mRNA molecules were purified from total RNA using Illumina's mRNA-seq sample prep kit and cDNA libraries were sequenced on a high-throughput Illumina (GAIIx) which generates about 35 million reads of 76 bases for each sample. These short reads were then aligned with the *P. pallidum* PN500 reference transcriptome (composed of the spliced sequences of all the transcripts annotated in the reference database) downloaded from the Social Amoeba Comparative Genome Browser (SACGB; http://sacgb.fli leibniz.de/cgi/index.pl). A total of 12,657 gene models (transcripts) are annotated from the genome of *P. pallidum* PN500. Approximately two third of the obtained reads mapped distinctively onto 12,657 annotated transcripts. Any read that could not be mapped to a unique sequence was not counted, thus eliminating repetitive elements. To estimate gene expression, each transcript was then quantified by calculating its RPKM (reads per kilobase of transcript per million mapped reads; Mortazavi *et al.* 2008). Using biological replicate I, 20,212,278 to 24,489,837 mappable reads were obtained, whereas biological replicate II generated between 16,943,246 and 20,182,623 mappable reads. Spearman correlation coefficients were calculated for all data sets. The biological replicates were highly comparable; when comparing each experimental condition, Spearman correlation coefficients were >0.95, demonstrating a high quality of data (Table 2). While comparing data from growing and starving cells, Spearman correlation coefficients ranged from 0.88 to 0.98 (Table 2). This difference signifies global changes in gene expression at the transition from growth to development.

	t0 exp1	t0 exp2	t2 exp1	t2 exp2	t3 exp1	t3 exp2	t2+ exp1	t2+ exp2	t3+ exp1	t3+ exp2
t0 exp1		0.950	0.934	0.895	0.938	0.904	0.938	0.897	0.939	0.912
t0 exp2			0.880	0.922	0.899	0.928	0.900	0.928	0.885	0.925
t2 exp1				0.953	0.984	0.949	0.979	0.938	0.977	0.949
t2 exp2					0.958	0.988	0.954	0.983	0.935	0.974
t3 exp1						0.968	0.982	0.948	0.986	0.969
t3 exp2							0.954	0.980	0.947	0.986
t2+ exp1								0.961	0.981	0.964
t2+ exp2									0.934	0.978
t3+ exp1										0.965
t3+ exp2										

Table 2: Spearman correlation coefficients for all RNA-seq data sets. The time t0 refers to vegetatively growing cells. Cells were staved for 2 or 3 hours without glorin treatment (t2, t3) or pre-starved for 1 hour and treated with glorin for additional 1 or 2 hours (t2+, t3+). exp: experiments (biological replicates).

To assess the differential regulation of genes by starvation and glorin treatment, analyses focused only on those gene transcripts that changed in expression at least 3-fold in each of the two biological replicates compared to a control condition to avoid noise in data.

3.6.1 Starvation triggers dramatic changes in gene expression in *P. pallidum* PN500 amoebae

To demarcate the effects of starvation from those of the glorin treatment, first, gene expression changes in response to starvation were studied using RNA-seq data from growing cells and from samples collected at 2 and 3 hours of starvation in buffer (with no glorin treatment). These analyses demonstrated that a considerable fraction of the genome of *P. pallidum* PN500 is represented as stage-specific transcripts. Many genes were found to be transcribed in vegetative cells, while others were transcribed at some time during early development. A number of transcripts found in cells at 3 hour of starvation were not found in vegetative cells, and many of the vegetative transcripts were no longer present by 3 hour of development (Appendix Table A3).

While comparing growing cells with cells developed for 2 hours, a total of 12,628 genes were found to be transcribed (Figure 22). 1,299 gene transcripts were seen to be differentially regulated after 2 hours of starvation; 911 transcripts were up-regulated and 388 transcripts down-regulated. Analysis of data from growing cells and cells starved for 3 hours revealed that 680 transcripts were up-regulated and 376 were down-regulated (Figure 22; Appendix Table A3). When a comparison was made between the lists of gene transcripts differentially regulated at 2 and 3 hours of starvation, it was found that 553 of the up-regulated genes and 283 of the down-regulated genes matched between two lists (Appendix Table A3).

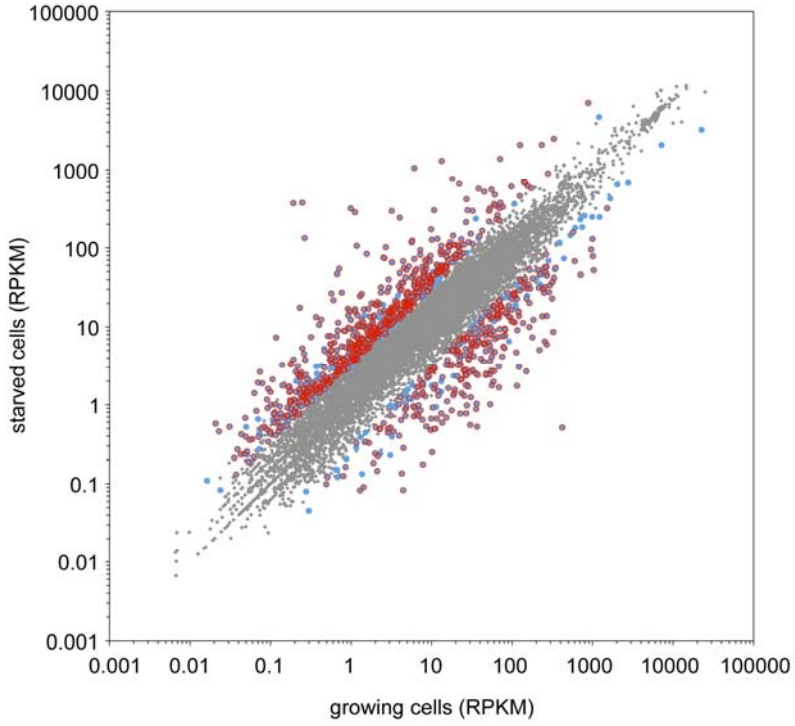


Figure 22: Effect of starvation on global gene expression in starving *P. pallidum* cells. A comparison is shown between steady-state levels of cellular transcripts (expressed as RPKM) in growing and starved cells for all 12,627 gene models (grey symbols). Genes that are regulated at least 3-fold in cells starved for 2 hours are denoted with blue dots with red circles. 911 genes were up-regulated and 388 down-regulated after 2 hours of starvation. Blue dots indicate differentially expressed genes in cells starved for 3 hours; 680 genes were up-regulated and 376 were down-regulated. Scatter blot presents the average result from two independent replications.

Starvation induced changes in expression of individual genes were highly reproducible when comparing RNA-seq data from both biological replicates (Table 2). The expression measurements obtained by RNA-seq analyses were further verified by quantitative RT-

PCR. As shown by absolute expression data (Appendix Table A3), expression of many genes, of which PPL_02780 and PPL_02774 are clear examples, increased immediately upon starvation and continued to increase to high levels linearly during the first 3 hours of development. PPL_02780 and PPL_02774 were then selected for confirmation of RNA-seq data using real-time RT-PCR. Quantitative RT-PCR results were in agreement with those obtained by RNA-seq analysis, thereby demonstrating validity of RNA-seq data (Figure 23).

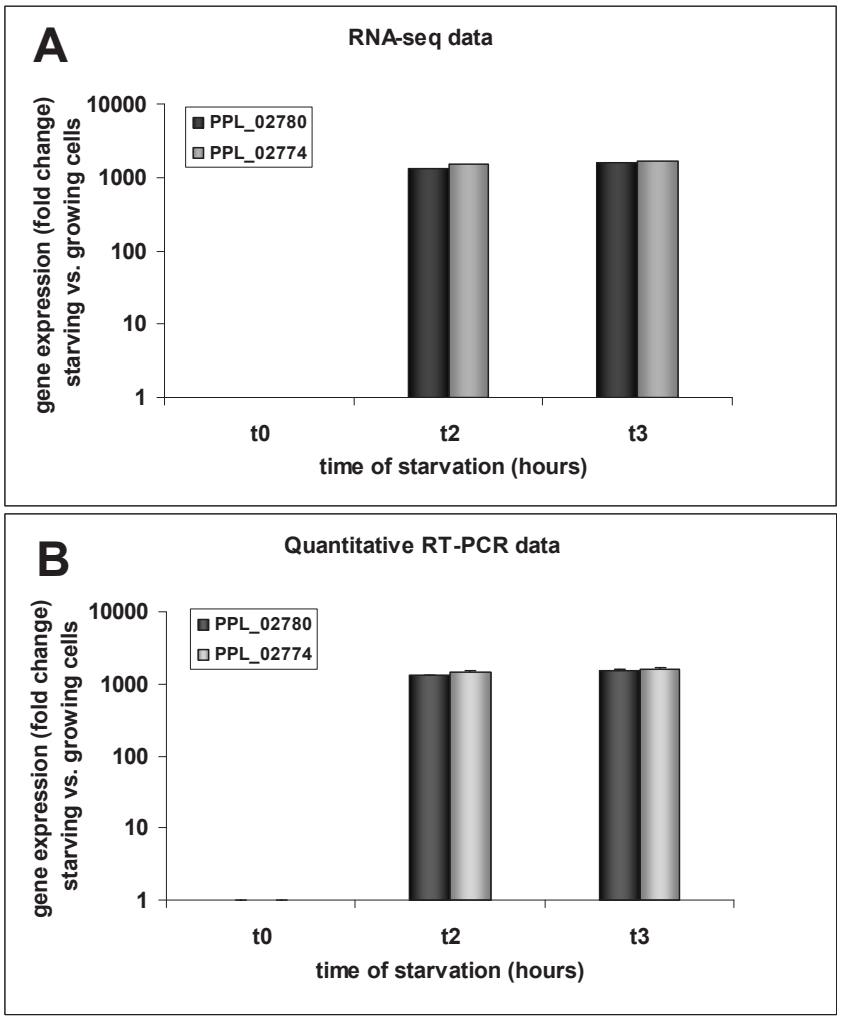


Figure 23: Correlation of RNA-seq data with real-time RT-PCR data. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 amoebae (**t0**; fold change set to 1). Values > 1 represent higher expression of the gene in starving cells than in growing cells. As a control, expression of house keeping gene *gpdA* was examined. Cells were starved in shaking cultures for 2 or 3 hours in the absence of exogenous glorin treatment (**t2, t3**). **(A)** Two model genes, indicated by the corresponding *P. pallidum* (PPL) gene numbers, were selected from RNA-seq data of first biological replicate to describe starvation induced gene expression**.** It should be noted that the results obtained from RNA-seq of second biological replicate were comparable to the results shown here. **(B)** Relative expression of representative genes (as shown in panel A) determined by real-time RT-PCR. cDNA used in this experiment were prepared from total RNA

extracted from first biological replicate. Mean values of triplicate measurements of the same cDNA ± SD were plotted. A high degree of correlation was observed between two methods demonstrating the effectiveness of RNA-seq approach.

Moreover, it was found that 837 genes diffrentially regulated by starvation (more than 3-fold) after 2 hours of starvation were similarly regulated one hour later (Appendix Table A3). This set of genes is identified as 'starvation-responsive genes' and symbolizes the growth arrest of the cells. Genes down-regulated by starvation are possibly those that are expressed during vegetative growth and shut off at the onset of starvation. Whereas genes up-regulated by starvation represent 'developmentally induced genes'. RPKM values and fold changes for each individual gene regulated at 2 and 3 hours of development are available in Appendix Table A3.

Overall, these results indicate that the transition of *P. pallidum* PN500 amoebae from growth-to-starvation is accompanied by predominant changes in the gene expression patterns.

3.6.2 Glorin-induced developmental regulation of gene expression

After getting an insight into starvation triggered gene expression changes, it was interesting to investigate whether exogenous glorin can stimulate differential changes in the early developmental gene expression. To explore this question, gene expression data from cells starved for 2 hours without glorin treatment were compared with the data from cells that were pre-starved for 1 hour and then treated with 1 µM final concentration of glorin at 30-minute intervals for an additional hour, meaning that only two pulses of glorin were applied. It was observed that stimulation of starving cells with glorin resulted in more than 3-fold differential expression of 115 gene transcripts, 70 of which were up-regulated by 3- to 57-fold (Figure 24A). When gene expression profiles of cells starved for 3 hours were compared with gene expression data of cells pre-starved for 1 hour and treated with 1 µM glorin every 30 minutes for 2 hours, a relatively different outcome was noticed (Figure 24B). In these analyses, 120 genes were differentially regulated more than 3-fold, but only 20 of them were up-regulated. Among these 20 genes, 11 were those that were induced more than 3-fold after 1 hour of glorin treatment also.

The phenomenon that many genes differentially up-regulated more than 3 fold after 1 hour of glorin treatment exhibited abrupt changes in expression after 2 hours of stimulation with glorin, could be explained by comparing the absolute abundance and

expression kinetics of these genes under starving conditions (in absence of glorin) as depicted in Appendix Table A4 & A5.

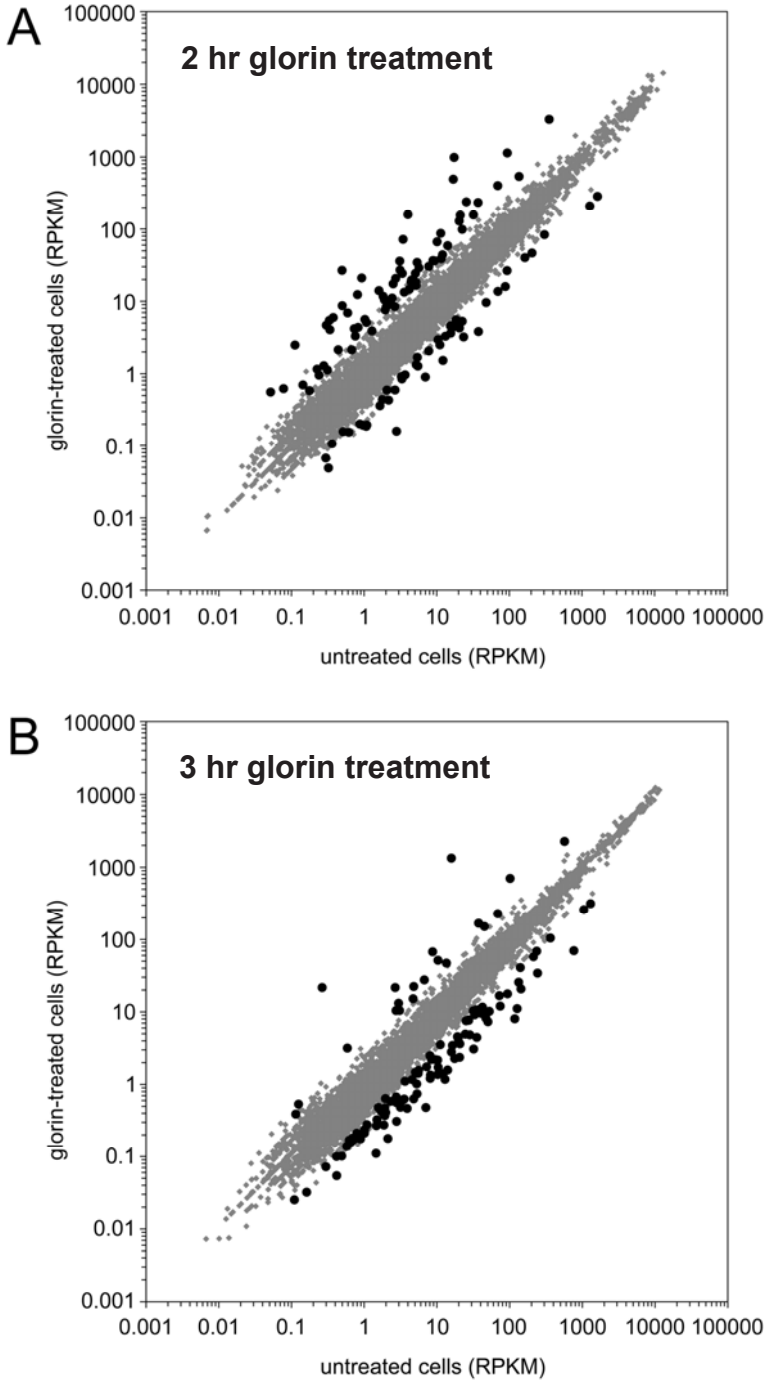


Figure 24: Effects of exogenous glorin stimulation on global gene expression in *P. pallidum* PN500. A comparison is shown between steady-state levels of cellular transcripts (expressed as RPKM) in *P. pallidum* PN500 cells treated with glorin for 1 hour (**A**) or 2 hours (**B**) and cells starved without glorin treatment for the time period. All 12,627 gene models are plotted (represented by grey symbols). Genes that were differentially regulated at least 3-fold after 1 or 2 hours of glorin treatment are indicated by black dots. Treatment of starving cells with glorin for 1

hour resulted in differential expression of 115 genes, 70 of which were up-regulated. After 2 hours of stimulation with glorin, 120 genes were differentially regulated, but only 20 of them were up-regulated more than 3-fold. Scatter plot presents the average result from two independent replications.

A few genes, of which PPL_05354 and PPL_09347 (Table 3; Figure 25) are obvious representatives, exhibited very low basal expression in growing cells and retained low level expression constantly during the first hours of starvation. In comparison, the majority of other genes (43 out of 70 genes up-regulated more than 3 fold after 1 hour of glorin treatment) showed a moderate increase in expression at the growth-to-starvation transition, possibly because cells gain the ability to set up endogenous glorin signalling within first few hours of starvation, however, their expression was further enhanced by exogenous glorin pulses (examples in Table 3 & Figure 27). Previously, it was shown that slow shaken high cell density cultures of *D. discoideum* were able to generate normal endogenous cAMP signals without exogenously supplied cAMP pulses (Kimmel & Carlisle 1986).

While the expression levels of these genes increased with the progression of starvation, the effects of glorin treatment became relatively less significant. As a result, many genes that were differentially induced by glorin more than 3-fold after 1 hour of glorin treatment were up-regulated less than 3-fold one hour later even in the presence of glorin. This demonstrates that externally added glorin caused the induction of genes precociously compared to the natural conditions of development. RPKM values and fold changes for each individual gene regulated by glorin at 2 and 3 hours of development are presented in Table 3 & Appendix Table A4, A5 and A6.

These data support the hypothesis that in starving *P. pallidum* PN500 amoebae, extracellular glorin binds to cell surface receptors, a signal is transduced intracellularly that leads to changes in developmental gene expression.

Table 3: Absolute expression data of selected genes obtained by RNA-seq analysis. The time t0 refers to vegetatively growing cells. Cells were staved for 2 or 3 hours without glorin treatment (t2, t3) or pre-starved for 1 hour and treated with glorin for additional 1 or 2 hours (t2+, t3+).

Gene Number	t0 RPKM	t2 RPKM	t3 RPKM	t2+ RPKM	t3+ RPKM	t2/ t0 fold change	t3/ t0 fold change	t2+/ t0 fold change	t3+/ t0 fold change	t2+/ t2 fold change	t3+/ t3 fold change
PPL_05833	0.42	3.50	6.56	56.75	10.97	8.21	15.41	133.2	25.75	16.21	1.67
PPL_03784	0.16	0.74	0.79	3.35	1.30	4.51	4.84	20.40	7.96	4.51	1.64
PPL_00912	0.60	0.76	1.16	9.54	2.66	1.27	1.93	15.87	4.42	12.40	2.28
PPL_05195	3.71	42.36	84.83	9.46	5.42	11.39	22.81	2.54	1.45	0.22	0.06
PPL_05702	13.94	17.65	24.43	1.72	10.97	1.26	1.75	0.12	0.78	0.09	0.44
PPL_04459	18.55	8.22	4.62	12.50	22.54	0.44	0.24	0.67	1.21	1.52	4.87
PPL_03541	0.08	0.065	0.069	0.81	0.069	0.75	0.80	9.44	0.80	12.55	0.99
PPL_11763	0.62	0.86	0.92	11.40	2.71	1.38	1.49	18.31	4.36	13.18	2.92
PPL_02774	0.21	320.8	361.1	188.3	478.5	1488.	1675.	874.0	2220	0.58	1.32
PPL_07847	202.2	0.49	0.22	0.29	0.22	0.002	0.001	0.001	0.001	0.585	0.99
PPL_12271	31.53	10.77	25.60	105.4	26.19	0.34	0.81	3.34	0.83	9.78	1.02
PPL_12249	22.37	10.59	4.54	273.1	48.53	0.47	0.20	12.20	2.16	25.77	10.66
PPL_12248	4.23	1.93	1.48	68.90	10.42	0.45	0.35	16.26	2.46	35.62	7
PPL_09347	0.10	0.24	0.26	15.18	13.27	2.25	2.42	139.4	121.9	61.76	50.31
PPL_07908	0.12	105.0	97.31	22.54	19.10	854.4	791.1	183.2	155.3	0.21	0.19
PPL_05354	21.33	22.40	18.61	1141	1682	1.05	0.87	53.75	79.23	50.95	90.37
PPL_03564	6.05	3.12	4.13	27.04	7.01	0.51	0.68	4.46	1.15	8.65	1.69
PPL_08455	8.58	0.33	0.53	4.05	1.07	0.03	0.06	0.47	0.12	11.98	2.02
PPL_08454	0.30	0.37	0.45	5.96	1.20	1.23	1.5	19.86	4	15.86	2.66
PPL_00855	0.20	1.72	2.66	2.99	5.33	8.6	13.3	14.95	26.65	1.73	2.00
PPL_04108	1.83	6.79	7.58	22.55	7.04	3.71	4.14	12.32	3.84	3.32	0.92
PPL_05727	59.16	14.16	18.86	58.89	19.59	0.23	0.31	0.99	0.33	4.15	1.03
PPL_00902	66.97	70.05	96.22	192.5	148.7	1.04	1.43	2.87	2.22	2.74	1.54
PPL_06644	0.77	0.50	0.58	8.70	3.17	0.64	0.75	11.29	4.11	17.40	5.40

3.6.2.1 Classification of glorin-induced developmental gene expression

For the convenience of data interpretation, genes differentially regulated by glorin were sorted into 5 classes on the basis of their expression kinetics. Instead of presenting the entire list of glorin-regulated genes in this section, some model genes were choosen to explain the glorin mediated modulation of gene expression.

3.6.2.1.1 Class I: Genes stably induced by glorin

RNA-seq data analyses indicated that glorin causes precocious and high-level induction of many genes (Table 3 & Appendix Table A4, A5, and A6). Prominent examples of such genes are PPL_09347 and PPL_05354. As described in the absolute RNA-seq data of glorin-regulated genes (Table 3), in vegetative-stage cells and also at the beginning of development (2 and 3 hours of starvation), transcripts of PPL_09347 were barely detectable, whereas PPL_05354 was expressed at very low levels under these conditions. Marked induction in gene expression was observed in response to exogenous glorin as shown in Figure 25.

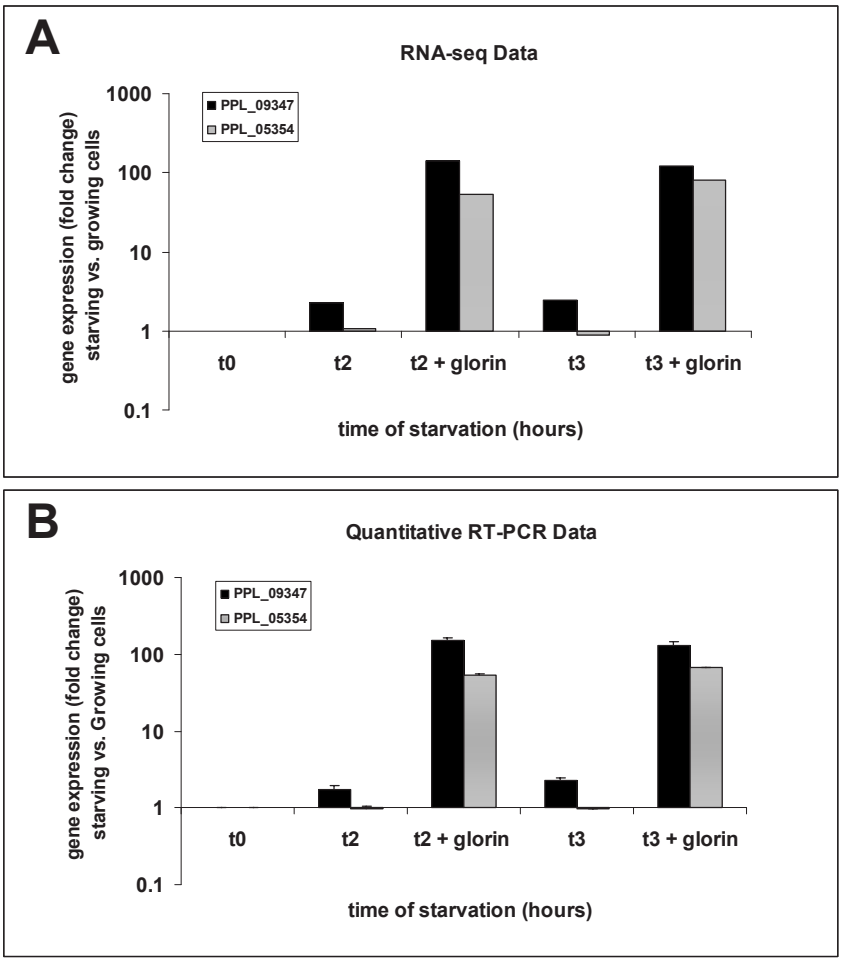


Figure 25: Correlation of RNA-seq data with real-time RT-PCR data. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 amoebae (**t0**; fold change set to 1). Values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of gene is lower in starving cells than in growing cells. As a control, expression of house keeping gene *gpdA* was examined. Cells were starved in shaking cultures for 2 or 3 hours in the absence of exogenous glorin treatment (**t2, t3**) or for 1 hour followed by 1 hour or 2 hours of glorin treatment (**t2+glorin, t3+glorin**). **(A)** Two model genes, indicated by the corresponding *P. pallidum* (PPL) gene numbers, were selected from RNA-seq data of first biological replicate to describe glorin-induced gene expression**.** It should be noted that the results obtained from RNA-seq of the second biological replicate were comparable to the results shown here. **(B)** Relative expression of representative genes (as shown in panel A) determined by real-time RT-PCR. cDNA used in this experiment were prepared from total RNA extracted from first biological replicate. Mean values of triplicate measurements of the same cDNA ± SD were plotted. A high degree of correlation was observed between two methods demonstrating the effectiveness of RNA-seq approach.

Transcripts of these genes accumulated rapidly in cells developed in buffer and treated with 1 µM of glorin at 30 minute intervals for for 1 or 2 hours. Induction of expression of these genes by glorin was maintained at high levels over the course of observation (Figure 25). In cells treated with glorin for 1 and 2 hours, level of expression of PPL_09347 was found to be 53- and 82-fold higher, respectively, than in vegetative cells.

PPL_05354 was induced 56- and 84-fold after 1 and 2 hours of glorin stimulation, respectively. This class of genes may exemplify ‘aggregation stage specific genes’. The expression measurements obtained by RNA-seq data analyses were validated by quantitative RT-PCR (Figure 25).

3.6.2.1.2 Class II: Genes transiently induced by glorin

When *P. pallidum* PN500 cells were developed in buffer suspensions and stimulated with glorin, many genes showed a transient increase in expression relative to the expression in growing cells. PPL_11763, PPL_12271 and PPL_03541 were rapidly induced by glorin followed by a rapid turn over leading to a sharp decline in expression level at the next hour of glorin treatment (Table 3; Figure 26). Transcripts of PPL_11763 and PPL_03541 were not expressed at significant levels in vegetative cells (Table 3) and cells developed for 1 or 2 hours in the absence of glorin treatment. After 1 hour of glorin treatment expression of these genes increased to 22.66- and 22.2-fold, respectively, compared to vegetative cells. In comparison, PPL_12271 was moderately expressed in growing cells (Table 3), also at the beginning of development, i.e. 1 hour of starvation but further development without glorin pulses led to a small decrease in the transcript level (Table 3; Figure 26).

Pulsing with glorin for 1 hour significantly upregulated this gene to 7.68 fold compared to growing cells. A rapid decline in expression of all these genes was observed after 2 hours of glorin treatment (Figure 26). These glorin-induced genes may represent a class of ‘developmentally induced aggregation specific genes’ with the exception of PPL_12271 that may also play some role in growing cells because adequate level of transcripts of this gene were detected in vegetative cells (Table 3). Real-time RT-PCR analysis confirmed the expression levels obtained in RNA-seq experiments (Figure 26).

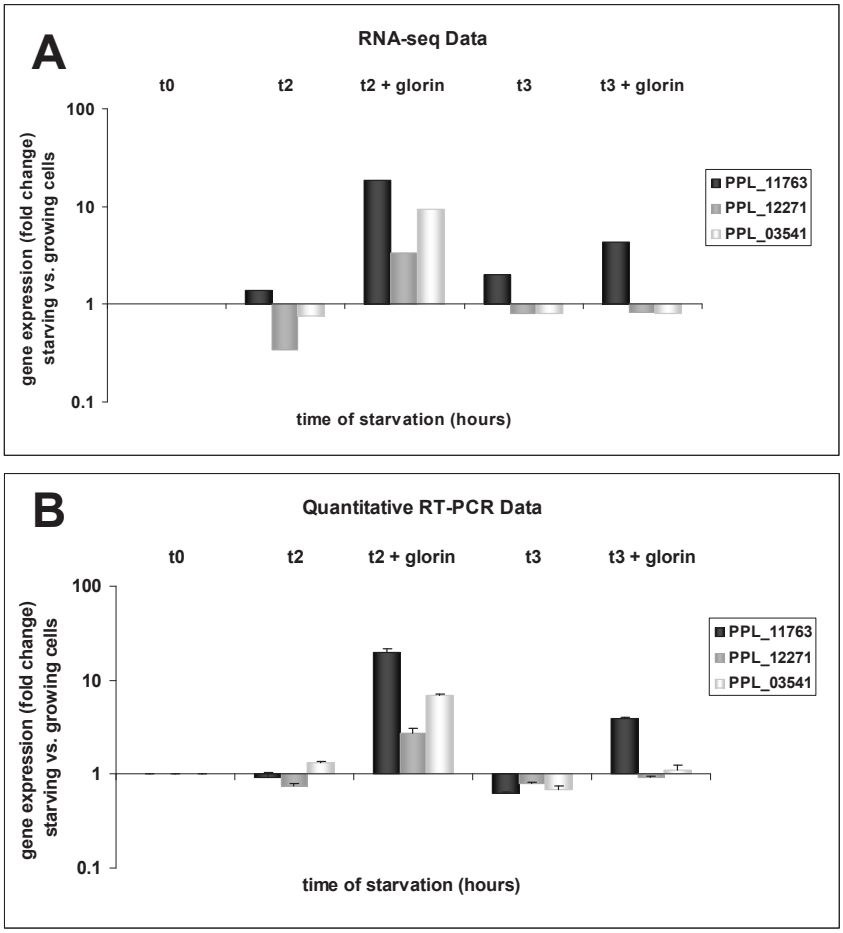


Figure 26: Correlation of RNA-seq data with real-time RT-PCR data. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 amoebae (**t0**; fold change set to 1). Values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of the gene is lower in starving cells than in growing cells. As a control, expression of house keeping gene *gpdA* was examined. Cells were starved in shaking cultures for 2 or 3 hours in the absence of exogenous glorin treatment (**t2, t3**) or for 1 hour followed by 1 hour or 2 hours of glorin treatment (**t2+glorin, t3+glorin**). **(A)** Two model genes, indicated by the corresponding *P. pallidum* (PPL) gene numbers, were selected from RNA-seq data of first biological replicate to describe glorin-induced gene expression**.** It should be noted that the results obtained from RNA-seq of second biological replicate were comparable to the results shown here. **(B)** Relative expression of representative genes (as shown in panel A) determined by real-time RT-PCR. cDNA used in this experiment were prepared from total RNA extracted from first biological replicate. Mean values of triplicate measurements of the same cDNA ± SD were plotted. A high degree of correlation was observed between two methods demonstrating the effectiveness of RNA-seq approach.

3.6.2.1.3 Class III: Genes induced by starvation, whereas glorin treatment further enhanced their expression

For some other genes, such as PPL_05833, PPL_03784 and PPL_00912, only low levels of transcripts were detected at 0 hr (Table 3). Starvation induced a moderate increase in the expression of PPL_05833 and PPL_03784 at 2 and 3 hours of development in buffer, whereas PPL_00912 was expressed at insignificant levels at 1 hour of starvation and only slightly upregulated at third hour of development in the absence of glorin pulses (Figure 27). When cells were exposed to glorin for 1 hour, transcripts of these genes accumulated significantly. PPL_05833, PPL_03784 and PPL_00912 were upregulated by 133-, 20- and 15-fold, respectively, compared to growing cells (Figure 27). Expression of PPL_03784 exhibited a low level of responsiveness to glorin. Comparatively reduced levels of expression were observed after 2 hours of glorin treatment indicating rapid turn over of these genes similar to class II genes. This class may symbolize ‘early developmental genes required shortly during aggregation’. Products of these genes may be needed during the first few hours of development.

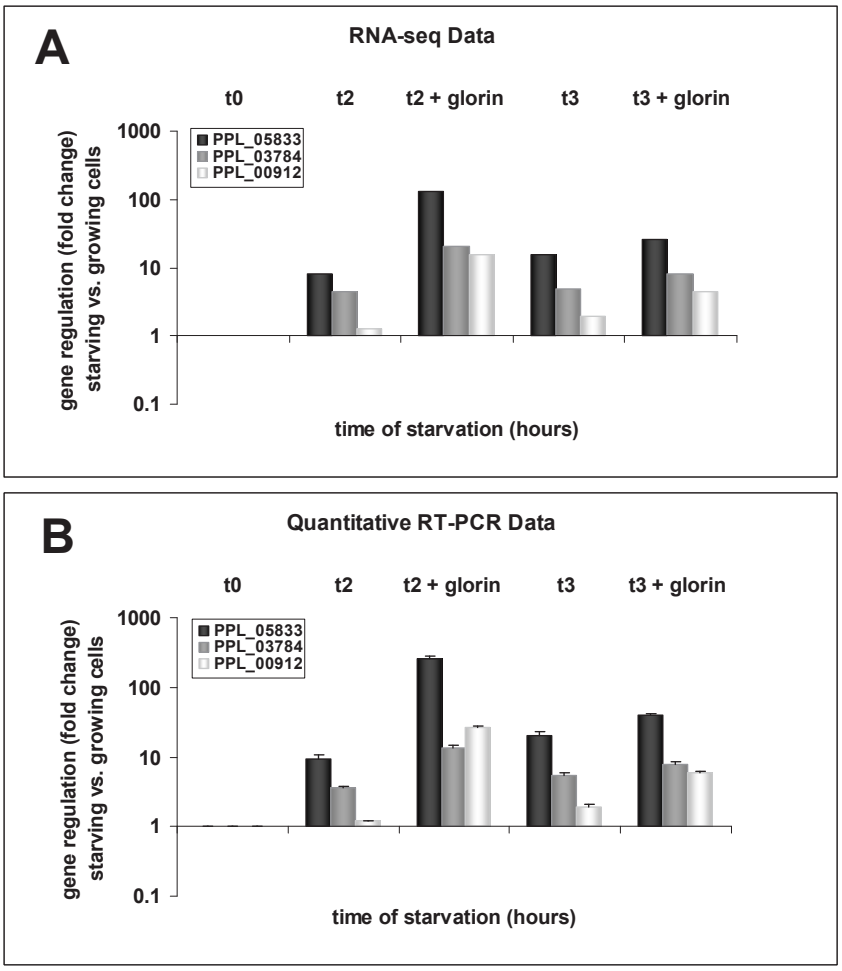


Figure 27: Correlation of RNA-seq data with real-time RT-PCR data. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 amoebae (**t0**; fold change set to 1). Values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of the gene is lower in starving cells than in growing cells. As a control, expression of house keeping gene *gpdA* was examined. Cells were starved in shaking cultures for 2 or 3 hours in the absence of exogenous glorin treatment (**t2, t3**) or for 1 hour followed by 1 hour or 2 hours of glorin treatment (**t2+glorin, t3+glorin**). **(A)** Two model genes, indicated by the corresponding *P. pallidum* (PPL) gene numbers, were selected from RNA-seq data of first biological replicate to describe glorin-induced gene expression**.** It should be noted that the results obtained from RNA-seq of second biological replicate were comparable to the results shown here. **(B)** Relative expression of representative genes (as shown in panel A) determined by real-time RT-PCR. cDNA used in this experiment were prepared from total RNA extracted from first biological replicate. Mean values of triplicate measurements of the same cDNA ± SD were plotted. A high degree of correlation was observed between two methods demonstrating the effectiveness of RNA-seq approach.

Induction of these genes by starvation indicates that some pre-starvation factor(s) may be involved in stimulating the expression of these genes. Glorin pulses further enhanced expression of this class of genes demonstrating that expression of these genes is controlled by a two-step regulatory pathway.

3.6.2.1.4 Class IV: Genes induced by starvation, whereas glorin treatment repressed their expression

Messenger RNA of many genes, represented by PPL_07908, PPL_05702 and PPL_05195 started to accumulate immediately at the onset of development; indicating that these are 'starvation-induced' genes. In vegetative cells, negligible amount of transcripts of PPL_07908 was present (Table 3). Starvation led to 854- and 791-fold increase in expression of this gene compared to vegetative cells, at 2 and 3 hours of development (Figure 28). Administration of exogenous glorin pulses elicited a rapid decline of expression, such that only 22.54- and 19.10-fold expression (compared to growing cells) was detectable after glorin treatment for 1 and 2 hours, respectively. In comparison, some transcripts of PPL_05702 were detected in vegetative cells, whereas starvation slightly increased expression of this gene (Table 3).

PPL_05195 exhibited basal level expression in growing cells (Table 3). A moderate increase in transcripts of this gene was observed at 2 hour of development, followed by a high level expression one hour later. As shown in the Figure 28, expression of PPL_05195 increased by 11.39- and 22.81-fold at 2 and 3 hour of starvation. Stimulating cells with glorin dramatically decreased expression level of this gene to 2.54- and 1.45-fold after 1 and 2 hours of treatment. RNA-seq data for expression of these genes was confirmed by real-time RT-PCR as illustrated in Figure 28.

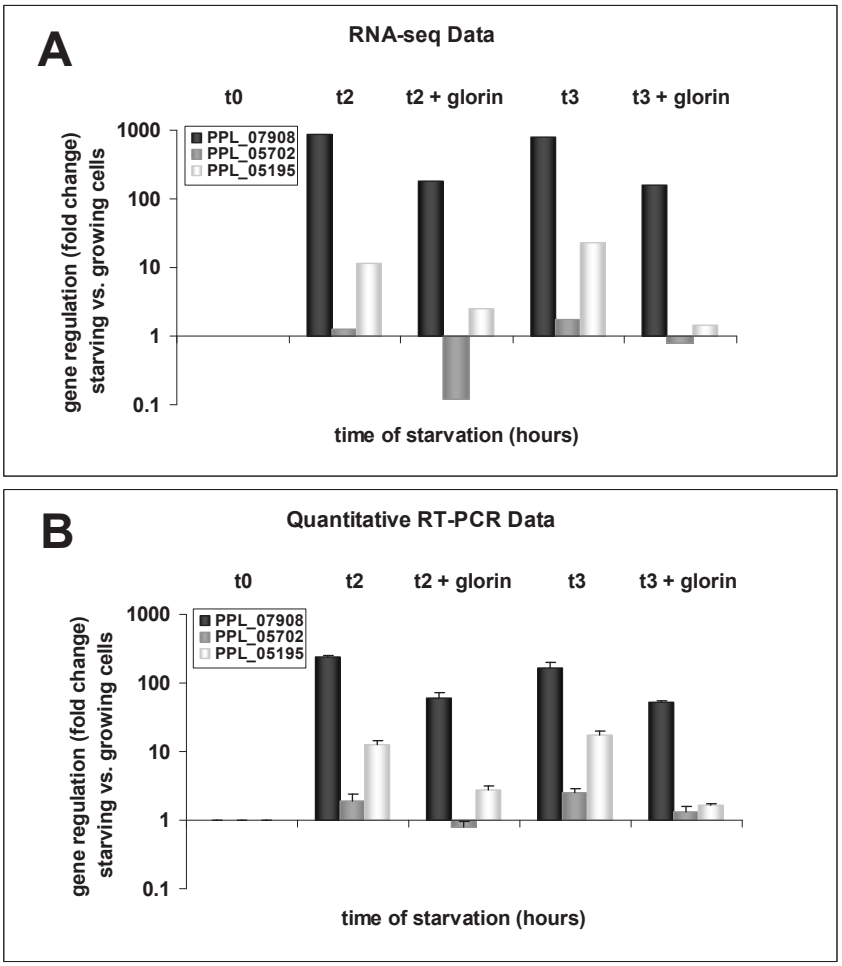


Figure 28: Correlation of RNA-seq data with real-time RT-PCR data. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 amoebae (**t0**; fold change set to 1). Values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of the gene is lower in starving cells than in growing cells. As a control, expression of house keeping gene *gpdA* was examined. Cells were starved in shaking cultures for 2 or 3 hours in the absence of exogenous glorin treatment (**t2, t3**) or for 1 hour followed by 1 hour or 2 hours of glorin treatment (**t2+glorin, t3+glorin**). **(A)** Two model genes, indicated by the corresponding *P. pallidum* (PPL) gene numbers, were selected from RNA-seq data of first biological replicate to describe glorin repressed gene expression**.** It should be noted that the results obtained from RNA-seq of second biological replicate were comparable to the results shown here. **(B)** Relative expression of representative genes (as shown in panel A) determined by real-time RT-PCR. cDNA used in this experiment were prepared from total RNA extracted from first biological replicate. Mean values of triplicate measurements of the same cDNA ± SD were plotted. A high degree of correlation was observed between two methods demonstrating the effectiveness of RNA-seq approach.

These genes represent 'differentially expressed early genes' and repression of their expression by glorin indicates that products of these genes may function only in the early hours of starvation and may not be required for aggregation. These genes are

considered to be negatively modulated by glorin pulses that may occur during the early development of *P. pallidum* to coordinate aggregation.

3.6.2.1.5 Class V: Genes repressed by starvation, whereas glorin treatment induces their expression

Expression of some other genes is negatively regulated during the early hours of development. Examples of such genes include PPL_12248 and PPL_12249. While considering absolute expression levels of these genes in growing cells of *P. pallidum* PN500, it was noticed that transcripts of PPL_12248 were present at low levels, whereas sufficient mRNA was detected for PPL_12249 (Table 3). With progression in development, transcripts of both genes declined in suspension developed cells.

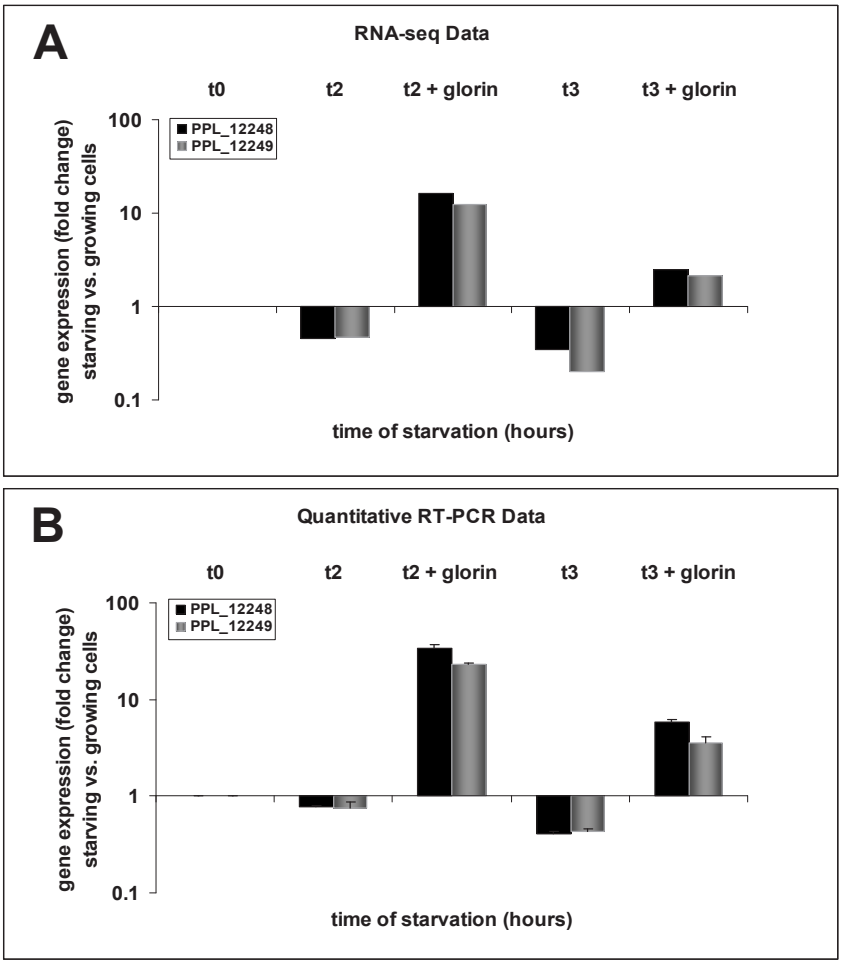


Figure 29: Correlation of RNA-seq data with real-time RT-PCR data. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 amoebae (**t0**; fold change set to 1). Values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1

show that expression of the gene is lower in starving cells than in growing cells. As a control, expression of house keeping gene *gpdA* was examined.Cells were starved in shaking cultures for 2 or 3 hours in the absence of exogenous glorin treatment (**t2, t3**) or for 1 hour followed by 1 hour or 2 hours of glorin treatment (**t2+glorin, t3+glorin**). **(A)** Two model genes, indicated by the corresponding *P. pallidum* (PPL) gene numbers, were selected from RNA-seq data of first biological replicate to describe glorin-induced gene expression**.** It should be noted that the results obtained from RNA-seq of second biological replicate were comparable to the results shown here. **(B)** Relative expression of representative genes (as shown in panel A) determined by real-time RT-PCR. cDNA used in this experiment were prepared from total RNA extracted from first biological replicate. Mean values of triplicate measurements of the same cDNA ± SD were plotted. A high degree of correlation was observed between two methods demonstrating the effectiveness of RNA-seq approach.

Development of cells in the presence of glorin led to an increase in mRNA levels of both PPL_12248 and PPL_12249. After 1 hour of glorin treatment, PPL_12248 was 16.26-fold induced compared to the growing cells, whereas PPL_12249 exhibited 12.20-fold increase in expression (Figure 29). Glorin-induced expression of both genes declined to low levels after stimulating cells with glorin for 2 hours indicating rapid turn over of these genes. RT-PCR data for validation of RNA-seq analyses of these genes is shown in Figure 29 that confirmed the RNA-seq findings.

PPL_12248 and PPL_12249 may represent ‘vegetative stage genes’ that turn off at the onset of development. Strong induction of these genes by glorin illustrates that products of these genes may also function in glorin signalling during aggregation of *P. pallidum* PN500 amoebae. Though these genes are transiently induced by glorin, they may play a crucial role if products of these genes are proteins required for only short period of time.

The results presented above clearly demonstrated that glorin may have two distinctly different effects during early development. The periodic addition of 1 µM glorin every 30 min stimulates the rate of expression of many genes, while expression of several other genes is suppressed. RT-PCR data used to evaluate performance of RNA-seq analyses indicated reliability of the experimental methods used in this study. These experiments also validate the experimental conditions used to in this study to examine glorin-regulated gene expression. Taken as a whole, studying differential gene expression changes mediated by glorin has contributed to the previous knowledge of cellular roles played by glorin.

3.7 Putative functions of glorin-regulated genes

To get insight into the possible roles of glorin responsive genes, putative molecular functions of these genes were searched based on homologies to annotated *D. discoideum*

genes (Social Amoebas Comparative Genome Browser; http://sacgb.fli-leibniz.de/cgi/index.pl). A short list of gene ontology (GO) terms attributed to the glorin-induced genes is presented in Table 4. Detailed information was obtained for genes significantly up- or down-regulated by glorin in three different organizing principles of GO, i.e. molecular function, biological process and cellular component (*P. pallidum* database; http://sacgb.fli-leibniz.de/cgi/index.pl) and is depicted in Appendix Table A7 and A8.

3.7.1 Annotation of GO terms to glorin-induced genes

Interestingly, it was found that a considerable number of genes induced by glorin may encode proteins that can play important roles in transducing extracellular signals. For example, the list encompasses seven G protein-coupled receptors, upregulated by glorin to various degrees. Several putative serine/threonine kinases are induced by glorin, some of which are alpha kinases carrying the conserved *"α-kinase catalytic domain"* as reported in *D. discoideum* myosin heavy chain kinases (MHCKs; Betapudi *et al.* 2005). The alpha-kinases family is a class of atypical protein kinases that exhibit low sequence similarity to conventional eukaryotic protein kinases. Some of these kinases contain von Willebrand factor A (vWFA) like motifs. The vWFA motif is found in a variety of intracellular proteins involved in various cellular functions, such as protein–protein interactions, DNA repair, transcription, ribosomal and membrane transport, and proteosomal functions (Betapudi *et al.* 2005; Whittaker and Hynes 2002). Some other proteins that include vWF domains take part in cell adhesion, signal transduction, migration, and pattern formation, engaging interaction with a variety of ligands (Colombatti *et al.* 1993). Extracellular signal-regulated kinase 2 (ErkB) is a protein involved in MAP kinase signal transduction pathways. *D. discoideum erkB* null mutants exhibit a decrease in motility and a severe chemotaxis defect towards cAMP gradient leading to impaired aggregation (Wang *et al.* 1998; Rodriguez *et al.* 2008). Stimulation of starving *P. pallidum* PN500 cells with glorin transiently upregulated expression of a gene designated as PPL_12271 that encodes putative ErkB protein. Glorin induced expression of genes encoding histidine kinases and tyrosine kinases-like transmembrane proteins that exhibit signal transduction activity across the cellular membrane.

A number of genes encoding small GTPases of Ras and Rho families were up-regulated by the glorin. Ras-superfamily small GTPases constitute components of signalling pathways connecting extracellular signals via transmembrane receptors to cytoplasmic or nuclear responses. Rho GTPases are involved in diverse signal transduction pathways,

including organization of the actin cytoskeleton during cell migration, activation of certain MAP kinases and may also activate transcription factors. Glorin induces a gene encoding a ‘Profilin I-like’ actin binding protein that may be involved in the dynamic turnover and restructuring of the actin cytoskeleton during chemotaxis.

Genes encoding zinc-binding domain containing proteins are also upregulated by glorin. One of these proteins is a RING zinc-finger domain holding protein. Zinc fingers are relatively small protein motifs that bind one or more zinc ions to help stabilize their fold. Zinc fingers usually consist of multiple finger-like protrusions that bind to the target molecule, such as DNA, RNA, proteins, or small molecules. Zinc finger-containing proteins play key roles in gene transcription, mRNA trafficking, translation, protein folding, cytoskeleton organisation, cell adhesion, and chromatin remodelling. A RING-finger domain is a protein structural domain of zinc finger type, whereas proteins containing a RING finger play vital roles in the ubiquitination pathway leading to degradation of proteins.

Glorin also induced expression of proteins involved in DNA metabolism, such as Deoxyribonuclease II that plays a major role in the degradation of nuclear DNA in cellular apoptosis during development. Glorin upregulated expression of genes encoding putative cytochrome P450 family proteins that are haem-containing mono-oxygenases, involved in oxidation-reduction processes. Enzymes of cytochrome P450 family play important role in the metabolism of compounds that control cell differentiation in *D. discoideum* (Gonzalez-Kristeller *et al.* 2008).

Glorin induced expression of many genes whose products are involved in carbohydrate metabolic processes. Examples include enzymes belonging to the glycoside hydrolase family 45 that exhibit endoglucanase activity, expansin-like proteins and cellulases. Expansins are unusual proteins that are believed to act as chemical grease and may allow cellulose fibrils to slide past one another when cellulose sheath is being assembled during the late aggregation stages. Similarly, cellulases seem to be involved in remodelling of slime sheath cellulose during morphogenesis (Sucgang *et al.* 2011).

Moreover, glorin induced genes encoding PA14 domain-containing proteins with homology to *D. discoideum* extracellular matrix proteins, such as EcmB. PA14 domain is a β-barrel structure recognized as a novel carbohydrate-binding module (Rigden *et al.* 2004).

The mannose 6-phosphate receptor homology (MRH) domain-containing family of proteins play key roles in the secretory pathway (Castonguay *et al.* 2011), whereas mannose 6-phosphate receptors (MPRs) play an essential role in lysosome biogenesis. These proteins (i.e. MPRs) bind newly synthesized Mannose-6-Phosphate-containing lysosomal hydrolases in the trans-Golgi network (TGN) and deliver them to the pre-lysosomal compartments (Castonguay *et al.* 2011).

Stimulation of cells with glorin induces expression of genes that may encode ABC transporter proteins belonging to family A & G. ATP-binding cassette (ABC) transporters are transmembrane proteins that make use of the energy of adenosine triphosphate (ATP) hydrolysis to transport various substrates across extra- and intracellular membranes, including metabolic products, lipids, toxins and drugs. Other genes induced by glorin may encode proteins displaying homology to glutamate-ammonia ligase involved in glutamine biosynthetic process, zymogen granule (pancreatic secretory granules) membrane glycoprotein, beta-lactamase-type transpeptidase fold containing protein, cellulose and chitin-binding domain containing proteins that exhibit carbohydrate-binding activity, epidermal growth factor-like domain containing membrane-bound protein implemented in cell recognition and proteins involved in proteolysis, pathogenesis and transcription repression.

The concomitant upregulation of these genes during early development suggests their possible involvement in the glorin-mediated coordination of aggregation process.

Gene	Expression (fold change)[a]	Gene description
Signal transduction		
G-protein coupled receptor activity		
PPL_08454	15.8	G-protein coupled receptor activity (family 3), metabotropic glutamate receptor-like
PPL_08455	11.9	G-protein coupled receptor activity (family 3), metabotropic glutamate receptor-like
PPL_03564	8.6	G-protein coupled receptor activity (family 3), metabotropic glutamate receptor-like
PPL_05727	4.1	G-protein coupled receptor activity (family 3), metabotropic glutamate receptor-like
PPL_04108	3.3	G-protein coupled receptor activity (family 3), metabotropic glutamate receptor-like
PPL_00902	2.7	G-protein coupled receptor activity (family 3), metabotropic glutamate receptor-like
PPL_00855	1.89	G-protein-coupled receptor (GPCR) family protein, frizzled and smoothened-like protein
protein serine/threonine kinase activity		
PPL_12248	39.6	protein serine/threonine kinase activity (alpha kinase superfamily)
PPL_12249	29.2	protein serine/threonine kinase activity (alpha kinase superfamily)
PPL_12251	8.7	protein serine/threonine kinase activity (alpha kinase superfamily); type A von Willebrand factor (VWFA) domain-containing protein
PPL_12250	4.76	protein serine/threonine kinase activity
PPL_12271	7.7	*erkB*, ERK subfamily protein kinase, extracellular response kinase
Tyrosine kinase-like protein		
PPL_00861	11.6	tyrosine kinase-like protein
Histidine kinase-like protein		
PPL_04384	4.11	histidine kinase, protein kinase, signal transducer activity, regulation of transcription
Ras GTPases		
PPL_07296	7.4	Ras GTPase domain-containing protein; type A von Willebrand factor (VWFA) domain-containing protein
Rho GTPases		
PPL_05452	5.8	Rho GTPase
PPL_04393	3.17	GTP binding activity, Rho GTPase, small GTPase mediated signal transduction
Cytoskeleton organization		
Actin cytoskeleton organization		
PPL_09347	53.9	profilin I, actin binding protein
Putative extracellular matrix proteins		
PPL_05354	56.9	putative extracellular matrix protein, PA14 domain-containing protein
PPL_12308	6.3	putative extracellular matrix protein, PA14 domain-containing protein
PPL_11304	2.8	putative extracellular matrix protein, PA14 domain-containing protein
Transcription and translation		
Zinc-binding domain-containing protein		
PPL_01619	3.32	CMP/dCMP deaminase, zinc-binding domain-containing protein
PPL_09669	4.37	RING zinc finger-containing protein
Secretory pathway		
protein binding function		
PPL_07812	15.4	Mannose-6-phosphate receptor domain, protein binding function
PPL_07800	5.48	Mannose-6-phosphate receptor domain, protein binding function

Transport		
ABC transporter family proteins		
PPL_07551	3.39	ABC transporter A family protein
PPL_07432	4.39	ABC transporter G family protein, integral to membrane
PPL_07551	3.49	ABC transporter A family protein, nucleoside-triphosphatase activity
Metabolism		
DNA metabolic process		
PPL_08854	2.84	similar to zymogen granule (pancreatic secretory granules) membrane glycoprotein, deoxyribonuclease II activity
PPL_05392	5.27	deoxyribonuclease II activity, DNA metabolic process
PPL_04328	3.40	DNA metabolic process, deoxyribonuclease II activity
Oxidoreductase activity		
PPL_09729	4.19	iron ion binding proteins, oxidoreductase activity
PPL_04071	1.21	electron carrier activity, iron ion binding activity
Carbohydrate metabolic process		
PPL_11763	22.6	expansin-like protein, Barwin-like endoglucanases, Glycoside hydrolase family 45 protein
Carbohydrate binding activity		
Cellulose-binding domain containing protein		
PPL_02674	2.7	Cellulose-binding domain containing protein, Carbohydrate binding domain-containing protein, Stalk-specific protein
PPL_05703	4.27	Cellulose-binding domain containing protein, Carbohydrate binding domain-containing protein, extracellular region
Cell recognition		
Epidermal Growth Factor-like domain containing membrane-bound proteins		
PPL_00062	3.3	Cell surface receptor, IPT/TIG Epidermal Growth Factor-like domain containing membrane-bound proteins
PPL_07302	4.90	Epidermal growth factor-like domain containing membrane-bound protein, calcium ion binding activity, extracellular in location
Miscellaneous		
Transcription repressor activity		
PPL_00612	3.95	transcription repressor activity
Proteolysis		
PPL_05027	3.90	ubiquitin carboxyl-terminal hydrolase 2, Peptidase C19, ubiquitin thiolesterase activity
PPL_08725	3.66	hydrolase activity, proteolysis
Pathogenesis		
PPL_10669	4.55	Delta endotoxin insectocide, pathogenesis
PPL_06262	3.80	delta-Endotoxin insectocide
Glutamate-ammonia ligase activity		
PPL_04550	3.73	glutamate-ammonia ligase, glutamine synthetase type I, glutamine biosynthetic process
beta-lactamase-type transpeptidase fold containing protein		
PPL_11763	22.6	beta-lactamase-type transpeptidase fold containing protein (based on homology to *D. discoideum* protein DDB_0189589.
Enhancing factor precursor		
PPL_04587	7.6	Enhancing factor precursor, Chitin-binding domain containing protein
Immunoglobulin E-set domain-containing protein		
PPL_04784	10.8	IPT/TIG domain-containing protein; immunoglobulin early-set domain-containing protein
PPL_04746	2.8	putative cell surface glycoprotein, IPT/TIG domain-containing protein, Immunoglobulin early-set domain-containing protein
Proteins with unknown function		
PPL_03541	22.2	unknown

PPL_05833	20.9	unknown
PPL_06644	17.4	unknown
PPL_07811	16.3	unknown
PPL_00117	12.1	unknown
PPL_00912	15.2	unknown
PPL_10324	11.6	unknown
PPL_02621	9.3	unknown
PPL_02620	9.3	unknown
PPL_07801	8.0	unknown
PPL_07818	7.2	unknown

Table 4: Summary of glorin-induced genes in *P. pallidum* PN500. [a]Fold change calculated for gene expression in cells prestarved for 1 hour and then treated with glorin for an additional hour, compared to gene expression in cells starved for 2 hours in the absence of exogenous glorin**.**

3.7.2 Annotation of GO terms to glorin-repressed genes

GO term analysis of glorin-repressed genes could not provide much information as most of these genes have not yet been ascribed to a distinct function. Genes that were downregulated by glorin may encode proteins that may be involved in the regulation of transcription, carbohydrate and lipid metabolic processes, proteolysis, pathogenesis, steroid metabolic processes, oxidoreduction reactions, nucleosome assembly and transportation.

Glorin treatment repressed expression of some genes whose products may exhibit O-methyltransferase activity. Methyltransferases play important role in many vital cellular processes including signal transduction, chromatin regulation, gene silencing, and protein repair. Expression of a gene that may encode myb transcription factor domain-containing protein was down-regulated by glorin. In *D. discoideum* Myb transcription factors play important role at culmination as activators of ancillary stalk differentiation (Tsujioka *et al.* 2007). Another gene repressed by glorin may encode a transcription factor including a homeodomain that binds DNA through a helix-turn-helix (HTH) structure.

Other glorin-repressed genes encode proteins with homology to cAMP-dependent protein kinase (PKA family protein kinase), winged helix-turn-helix transcription repressor, zinc ion binding proteins, and SecA protein that is a superfamily 2 helicase adapted to translocate proteins. GO annotation for some of the glorin-repressed genes is presented in Appendix Table A7 and A8.

3.8 Detailed kinetics of glorin-regulated gene expression

3.8.1 Effect of signal modulation on glorin-regulated gene expression

Previously, Kopachik (1990) studied protein synthesis by developing *P. violaceum* amoebae in response to externally added glorin and showed that this reaction was concentration and time dependent (Kopachik 1990). The author reported that a higher concentration of glorin added for a longer time was most effective to stimulate synthesis of certain proteins (Kopachik 1990). Therefore, it was interesting to elucidate effects of different concentrations and pulsing frequencies of glorin on gene expression changes in *P. pallidum* PN500 amoebae.

3.8.1.1 Gene expression analysis under different concentrations of glorin

First, starving *P. pallidum* PN500 cells were stimulated with varying concentrations of glorin and changes in gene expression were examined.

P. pallidum PN500 cells cultured on bacterial lawns were harvested and starved by slow shaking in phosphate buffer at a cell density of $2x10^7$cells/ml at 20°C. Before inducing development, $2x10^7$ vegetative stage cells were pelleted and stored at -80°C. This pellet of cells represented 'growing cells control' for total RNA extraction in subsequent gene expression analyses. After one hour of pre-starvation, culture was divided into 4 erlenmeyer flasks and cells were allowed to develop in shaking suspensions either in the presence or absence of glorin. Cells in each of three flasks were given glorin pulses at 10 nM, 100 nM and 1 µM final concentration, respectively, at 30-min intervals. Cells in one flask received no exogenous treatment (shown by '0 nM glorin' symbol in Figure 30) and served as the 'control' for the time period. Cells ($2x10^7$) were harvested from all four cultures at 0.5, 1 and 2 hours after glorin treatment for isolation of total RNA to prepare cDNA for gene expression analysis using real-time RT-PCR. Cell samples were collected from 'untreated control' at the same time points. Model glorin-regulated genes, PPL_09347 and PPL_05354 that were noticed to be highly induced by glorin in RNA-seq analyses were chosen to study gene expression changes in response to varying concentrations of glorin applied at 30- minute intervals. Relative gene expression levels of both genes under all tested conditions were calculated and fold changes were determined compared to expression in growing cells.

It was found that exposure of cells to 10 nM glorin for the first 30 minutes (one pulse only) was sufficient to induce PPL_09347 to 30.50-fold, compared to growing cells, whereas further stimulation of cells with 10 nM glorin added every 30 minutes for additional 1 and 2 hours increased expression of PPL_09347 by 39.26- and 45.62-fold, respectively (Figure 30A).

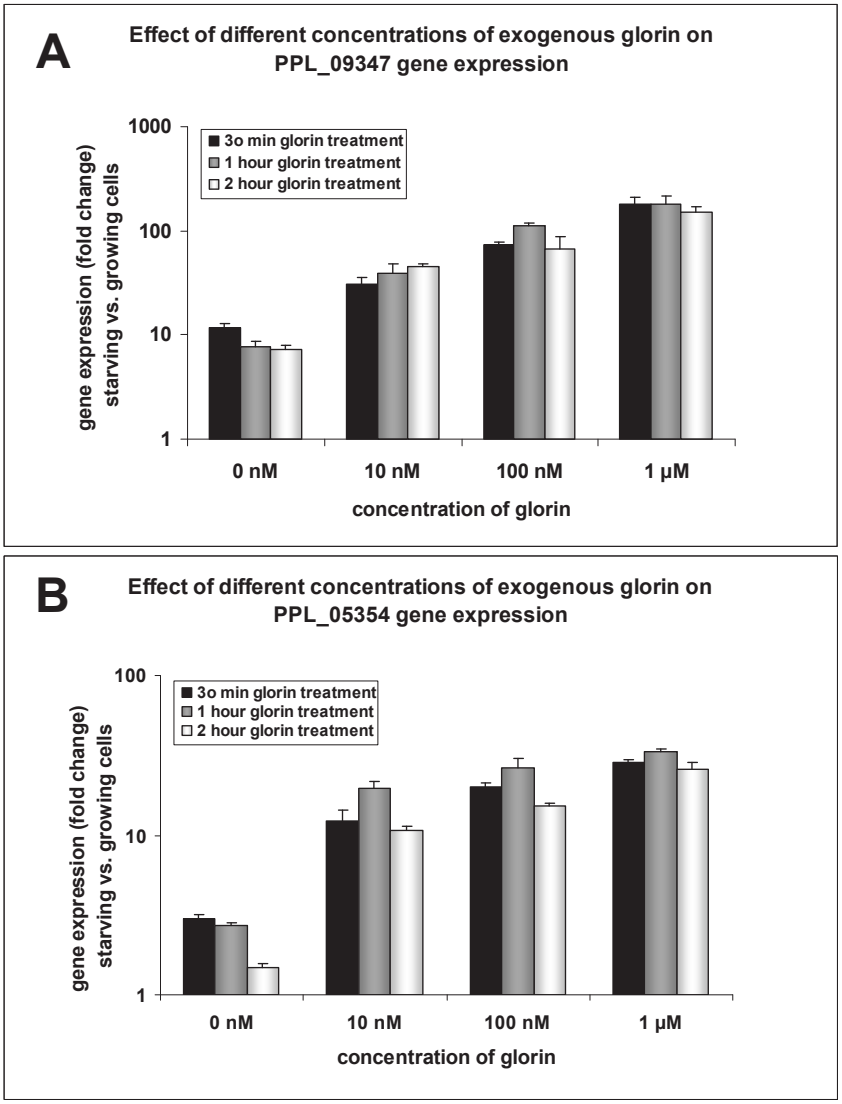


Figure 30: Glorin-induced gene expression in response to varying concentrations of glorin. *P. pallidum* PN500 cells were developed in shaking cultures for 3 hours in the presence or absence of exogenous glorin. In glorin treated cultures, beginning 1 hour after starvation, glorin was added at 10 nM, 100 nM or 1 µM final concentration at 30-minute intervals. Untreated control is represented by 0 nM glorin. Cell samples were collected after 30 min, 1 and 2 hours of glorin treatment for total RNA extraction to analyse gene expression. Cells were generally harvested 30 minutes after a pulse of glorin was applied. Samples were collected from untreated cells at the same time points. Relative expression of model genes PPL_09347 (A) and PPL_05354 (B) was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P.*

pallidum PN500 cells. Fold change was set to 1 where values > 1 represented higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of gene is lower in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

Treatment of developing cells with 100 nM glorin for 0.5-, 1- and 2-hours led to 72.49, 43.09- and 67.76-fold expression of PPL_09347. When 1 µM glorin was added at 30 minutes intervals to cells pre-starved for 1 hours, 180.15-, 178.13- and 150.79-fold increased in gene expression of PPL_09347 was observed after 30 minutes, 1 and 2 hours of glorin treatment. It is interesting to note that only after first pulse of 10 nM, 100nM, or 1 µM glorin (final concentration), PPL_09347 was induced to 30.50-, 72.49- and 180.15-fold respectively (Figure 30A). Overall, expression of PPL_09347 generally increased in response to glorin stimulation in the course of observation.

While analysing expression of model gene PPL_05354, it was noted that upon treatment of cells with 10 nM glorin every 30 minutes for 0.5-, 1- and 2-hours, gene expression increased adequately by 12.31-, 19.84-, and 10.71-fold, respectively (Figure 30B). Exposure of cells to 100 nM glorin for 30 minutes, 1 and 2 hours enhanced expression of PPL_05354 by 19.86, 26.12 and 15.15-fold, respectively. When glorin was added to starving cells at 1 µM final concentration, 28.39-, 33.52- and 25.67-fold increase in mRNA levels was noticed after 0.5-, 1- and 2-hours of treatment, while comparing expression to that in growing cells (Figure 30B). An important observation was the slight decrease in expression of PPL_05354 at 3 hours of development under all tested conditions. This may indicate that expression of PPL_05354 reduces to varying degrees with progression of starvation when cells are developed in shaking cultures.

Contrary to the results obtained in RNA-seq analyses (Figure 25), PPL_09347 and PPL_05354 exhibited a considerable increase in expression in cells that were not treated with glorin (referred to t2 and t3 in Figure 25 & untreated control ’0 nM’ in Figure 30). In experiment shown in Figure 25, untreated cells did not show significant expression of PPL_09347 and PPL_05354 genes. A possible interpretation of detecting low level expression of PPL_09347 and PPL_05354 in experiment depicted in Figure 30 could be that cells were harvested for this experiment when they were already starving on agar plates where they were grown in association with bacteria and most probably have already established some basic endogenous glorin signalling by themselves.

In short, it is shown that increasing concentrations of externally added glorin are associated with increased response in gene expression; maximum response in gene

expression was obtained when cells were stimulated with 1 μM glorin. Thus, these experiments further validated experimental conditions used for conducting RNA-seq analyses (described in Section 3.6). These data also provided an indication that glorin triggers rapid changes in gene expression.

3.8.1.2 Gene expression analysis under different pulsing frequencies of glorin

Physiological effects of many messengers of intercellular communication might be regulated by the frequency of their temporal variation (Li & Goldbeter 1992; Knobil 1981). Such a frequency encoding may manifest more advantages than amplitude-modulated control (Rapp 1987). Frequency encoding of signals is exemplified by secretion of cAMP pulses in aggregating *D. discoideum* cells at periodicities of ~6 min (McMains *et al.* 2008; Goldbeter 2006). To date pulsatile glorin signalling has not been observed. Previously some researchers showed that starving *P. violaceum* exhibit oscillatory aggregation, but it was pointed out that glorin may not be the primary oscillator (De Wit *et al.* 1988). Similarly, some initial reports described the presence of centrifugally propagated waves of excitation in the aggregation fields of *P. violaceum* amoebae that were regularly spaced with an interval decreasing from 4 to 2.5 minutes, while in *P. pallidum* amoebae, these waves were not found to be systematically spaced (Jones 1976).

As described in the Section 3.8.1.1, 1 μM final concentration of glorin was found to be optimal for studying glorin-induced gene expression. As a next step, gene expression changes in response to different pulsing frequencies of glorin were analyzed to understand how intervals between pulses may modulate gene expression.

P. pallidum PN500 cells were grown in association with bacteria and harvested after 48 hours of growth. Cells were washed and resuspended in phosphate buffer at a density of $2x10^7$cells/ml. A pellet of vegetative cells (~ $2x10^7$ amoebae) was collected for total RNA extraction for subsequent gene expression analyses. Amoebae were then starved in suspension culture for 1 hour by shaking at 100 rpm at 22℃ to induce development. After 1 hour pre-starvation, cell suspension was splitted into 4 parts. One culture designated as “untreated control” did not receive any exogenous treatment. Glorin (1 μM final concentration) was added for 2 hours to each of 3 other cultures at 10-, 20- and 30-minute intervals under slow-shake conditions (i.e. 100 rpm). Control culture was developed similarly in the absence of externally added glorin. Cell samples were

collected after 1 and 2 hours of glorin treatment to prepare total RNA for gene expression analysis. Samples were collected from 'untreated control' at the same time points.

To determine whether varying periods of exposure to glorin influenced gene expression, model genes PPL_09347 and PPL_05354 were subjected to quantitative RT-PCR analyses. Relative expression levels of both genes were determined under 3 different conditions of glorin treatment and fold changes were calculated compared to the expression in growing cells (Figure 31).

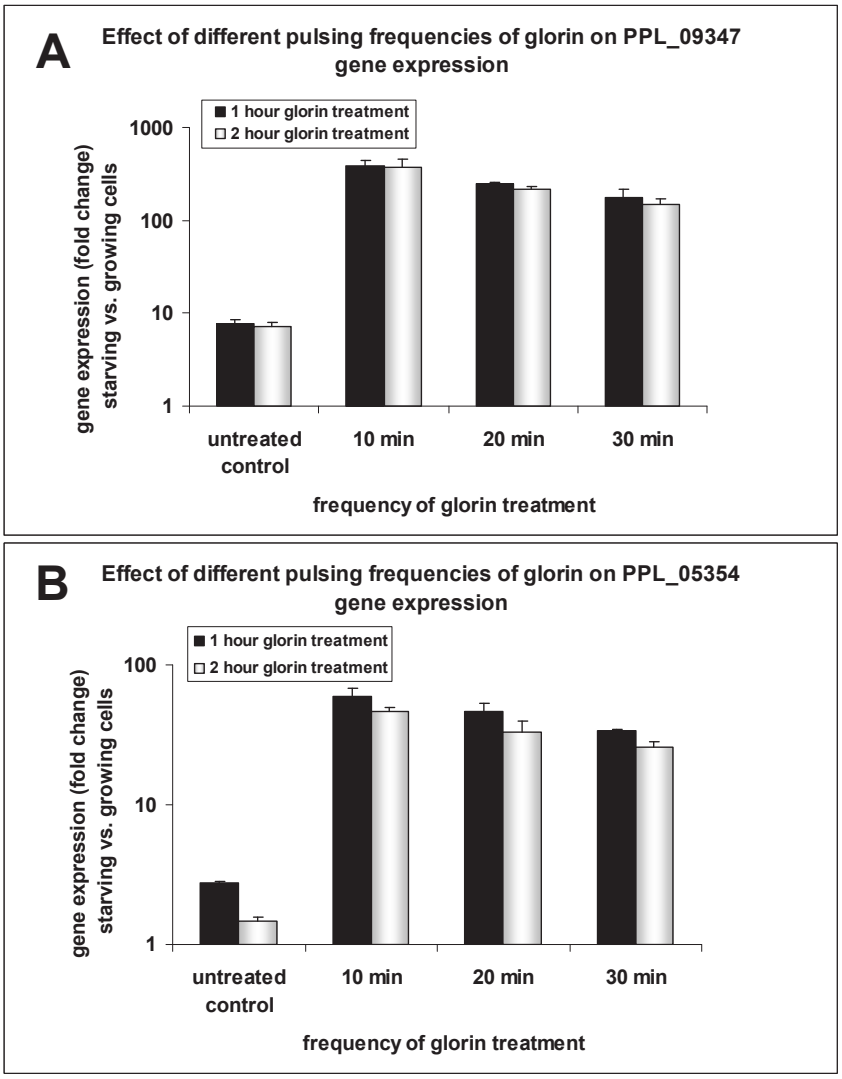


Figure 31: Glorin-induced gene expression in response to varying periods of exposure to glorin. *P. pallidum* PN500 cells were developed in shaking cultures for 3 hours in the presence or absence of exogenous glorin. In glorin treated cultures, beginning 1 hour after starvation, 1 µM final concentration of glorin was added at 10-, 20- or 30-minute intervals. Untreated control is shown seperately. Cell samples were collected after 1 and 2 hours of glorin treatment for total RNA extraction to analyse gene expression. Cells were generally harvested 30 minutes after a

pulse of glorin was applied. Samples were collected from untreated cells at the same time points. Relative expression of PPL_09347 (A) and PPL_05354 (B) was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of gene is lower in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

Real-time RT-PCR analysis showed that mRNA levels of PPL_09347 were highly up-regulated by 385- and 378-fold after 1 and 2 hour of glorin treatment at 10 minute intervals (Figure 31A). When starving cells were treated with 1 µM glorin at 20 minute intervals for 1 or 2 hours, PPL_09347 was induced to 249- and 212-fold, respectively, compared to growing cells. Adding glorin at 30 minute intervals led to 178- and 150-fold increase in expression of PPL_09347 at 1 and 2 hours (Figure 31A). For each pulsing condition tested, expression of PPL_09347 was similarly maintained at both hours of glorin treatment.

When gene expression data of PPL_05354 were analyzed, it was found that this gene was induced to 33.52- and 25.67-fold (compared to growing cells) when cells were pulsed with glorin at 30-minute intervals for 1 and 2 hour, respectively (Figure 31B). Significant level expression of this gene to 46.91- and 33.20-fold was noticed after pulsing cells with glorin at 20-minute intervals for 1 and 2 hours, respectively. Pronounced changes in gene expression levels were detected when cells received pulses of glorin at 10-minute intervals. 59.78- and 46.92-fold increase in expression of PPL_05354 was observed after 1 and 2 hours of exogenous glorin treatment, respectively (Figure 31B).

These data demonstrate that 1 µM glorin (final concentration) induced model genes significantly when pulsed at 30-minute intervals, whereas comparatively higher levels of gene expression were obtained when glorin pulses were applied more frequently at 10-minute intervals indicating that repetitive stimulation with glorin results in a stronger response. These results further support authenticity of pulsing conditions used for RNA-seq analyses. It still remains to uncover how often pulses of glorin are secreted in aggregation fields of starving *P. pallidum* PN500 amoebae.

3.8.2 Expression kinetics of glorin-regulated genes during the first 5 hours of development in suspension cultures

In the RNA-seq analysis and subsequent experiments, gene expression studies mainly focused on first 3 hours of starvation encompassing 2 hours of glorin treatment. After RNA-seq data analysis provided a list of glorin-regulated genes, and model genes were defined to study glorin effects on gene expression using real-time RT-PCR, it could be advantageous to investigate expression kinetics of glorin -induced and -repressed genes beyond 3 hours of starvation in the presence or absence of exogenous glorin using suspension cultures of *P. pallidum* PN500 amoebae.

Therefore, expression kinetics of selected glorin-regulated genes were determined during the first 5 hours of development under four different conditions. 5 hour duration was chosen in this experiment because it was assumed that within this time cells encounter a prominent shift from pre-aggregation to aggregation phase and may undergo major changes in gene expression patterns. *P. pallidum* PN500 amoebae were harvested in the late vegetative stage, washed to remove nutrients and suspended in phosphate buffer at a cells density of $2x10^7$cells/ml. The amoebae in suspension culture were pre-starved for 1 hour by slow shaking at 100 rpm at 22^oC to trigger development. After this initial treatment, cell suspension was equally divided into 5 parts. One culture of cells was not treated with glorin and acted as ‘untreated control’. Each of 4 other cultures received four different glorin treatments for additional 4 hours. To one culture, 1 µM of glorin was added at 10-minute intervals, whereas a second culture received 100 nM (final concentration) glorin every 10-minutes. The third culture was treated with 1 µM (final concentration) glorin at 30-minute intervals, while fourth culture was pulsed with 100 nM (final concentration) glorin at 30-minute intervals. All these cultures were then developed for 4 additional hours under slow shaking conditions. By this approach, the objective was to investigate how gene expression is modulated during development under suspension when starving cells are pulsed with low and high levels of glorin at varying intervals for 4 hours. $2x10^7$ cells were harvested from glorin treated cultures after 0.5-, 1-, 2-, 3-, and 4-hours of glorin treatment. Samples ($2x10^7$cells per sample) were collected from untreated control culture at the same time points. Samples were generally collected 30 minutes after a pulse of glorin was applied. Total RNA was extracted to prepare cDNA for gene expression analysis using real-time RT-PCR. Relative gene expression of model genes PPL_09347, PPL_05354, PPL_05833, PPL_12271 and PPL_07908 was determined and fold changes were calculated compared to the expression in untreated cells. Four hours

of glorin treatment represented total 5 hours of starvation (1 hour pre-starvation + 4 hours glorin treatment).

Analyzing expression data of glorin induced gene PPL_09347 under above mentioned conditions revealed that both 1 µM and 100 nM concentrations of glorin were equally effective to induce high level gene expression when cells were pulsed frequently, i.e. at 10-minute intervals (Figure 32). 1 µM glorin added every 30 minute also exhibited comparable induction of PPL_09347, whereas expression levels observed in response to 100 nM glorin added at 30-minute intervals were relatively lower, signifying the requirement for higher concentrations of glorin to achieve optimal induction of expression (Figure 32). PPL_09347 retained high levels of expression at all 4 hours of glorin treatment.

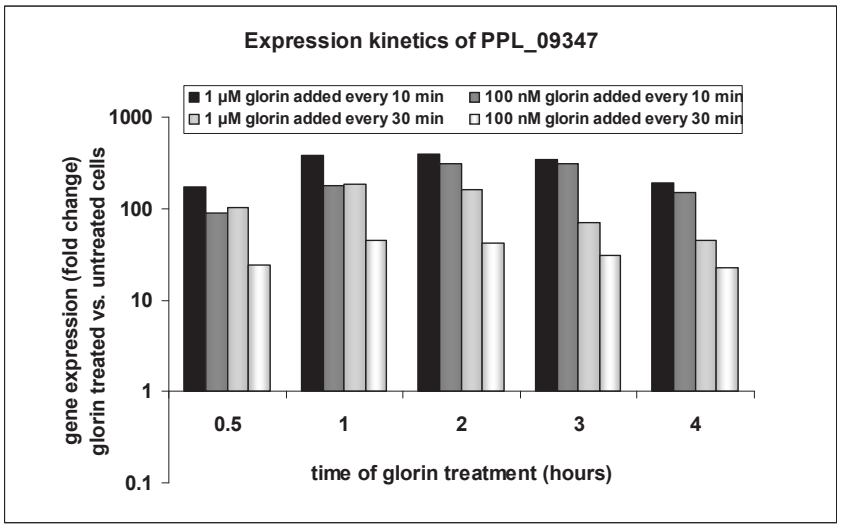


Figure 32: Glorin-induced changes in expression of PPL_09347 in response to varying concentrations and periods of exposure to glorin. *P. pallidum* PN500 were developed in shaking cultures for 5 hours in the presence or absence of exogenous glorin. In glorin treated cultures, beginning 1 hour after starvation, either 1 µM or 100 nM final concentration of glorin was added at 10 or 30-minute intervals. Cell samples were collected after 0.5-, 1-, 2-, 3- and 4-hours of glorin treatment for total RNA extraction to analyse gene expression. Cells were generally harvested 30 minutes after a pulse of glorin was applied. Samples were collected from untreated cells at the same time points. As a control, expression of house keeping gene *gpdA* was examined. Relative expression of model genes PPL_09347 was determined by real-time RT-PCR. Fold changes are shown. All data were compared to untreated *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represent higher expression of the gene in glorin treated cells than in untreated cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted. 4 hours of glorin treatment represented total 5 hours of starvation.

Another glorin-induced gene, PPL_05354, exhibited progressive increase in expression for the first 3 hours of glorin treatment, whereas expression levels declined drastically at 4th hour (Figure 33). At 4th hour of glorin treatment, low level expression of this gene was

still detectable in cells pulsed frequently at 10-minute intervals with 1 µM or 100 nM glorin, whereas PPL_05354 showed clear down-regulation in cells treated with 1 µM or 100 nM glorin at 30-minute intervals (Figure 33). These data provide an understanding of kinetics of PPL_05354 in slow shaking cultures. There is a gradual increase in gene expression during the first 3 hours of glorin treatment followed by a steep reduction in mRNA levels. This rapid decline in expression may be explained by suggesting that starving cells may secrete glorin by themselves after a few hours of development and accumulation of glorin (exogenous glorin + glorin secreted by cells) may result in autonomous signalling, therefore, effects of externally added glorin appear less prominent.

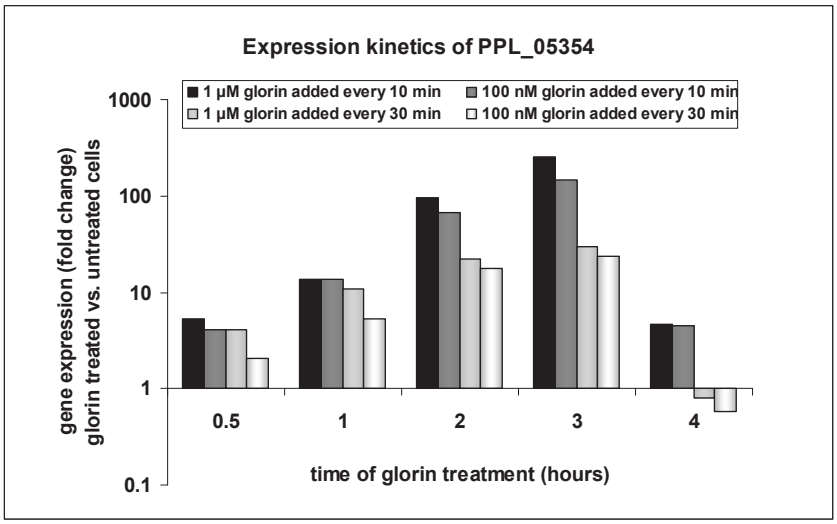


Figure 33: Glorin-induced changes in expression of PPL_05354 in response to varying concentrations and periods of exposure to glorin. *P. pallidum* PN500 were developed in shaking cultures for 5 hours in the presence or absence of exogenous glorin. In glorin treated cultures, beginning 1 hour after starvation, either 1 µM or 100 nM final concentration of glorin was added at 10 or 30-minute intervals. Cell samples were collected after 0.5-, 1-, 2-, 3- and 4-hours of glorin treatment for total RNA extraction to analyse gene expression. Cells were generally harvested 30 minutes after a pulse of glorin was applied. Samples were collected from untreated cells at the same time points. As a control, expression of house keeping gene *gpdA* was examined. Relative expression of model genes PPL_05354 was determined by real-time RT-PCR. Fold changes are shown. All data were compared to untreated *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represent higher expression of the gene in glorin treated cells than in untreated cells. Values < 1 show that expression of gene is lower in glorin treated cells than in untreated cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted. 4 hours of glorin treatment represented total 5 hours of starvation.

PPL_05833 was another gene chosen from RNA-seq data of glorin-induced genes. This gene manifested very unique kinetics. It was highly induced within 30 minutes of glorin treatment, followed by progressive decline, such that only basal level expression was

detectable after 4 hours of glorin treatment (Figure 34). These data were in agreement with the RNA-seq results which indicated that PPL_05833 exhibits rapid turn over and high-level gene expression is followed by a prompt decline. This phenomenon may be similarly explained as in case of PPL_05354 (Figure 33) by proposing that setting up of endogenous glorin signalling by starving cells may result in less significant responses to externally added glorin. Yet, a second possiblity is that product of PPL_05833 might be required only for very short period of time during aggregation, therefore expression of this gene declines quickly. However, it can not be ignored that mRNA of this gene might be unstable under shaking culture conditions leading to rapid decline in transcript levels. Induction of PPL_05833 in response to 1 µM and 100 nM glorin pulsed at 10-minute intervals was comparable and a high-level expression was detected under both of these conditions (Figure 34). Significant increase in gene expression was observed when cells were treated with 1 µM glorin at 30-minute intervals. Messenger RNA of PPL_05833 accumulated to relatively lower levels in response to pulses of 100 nM glorin delivered at 30-minute intervals manifesting need of frequent pulsing with glorin to detect optimal gene expression responses.

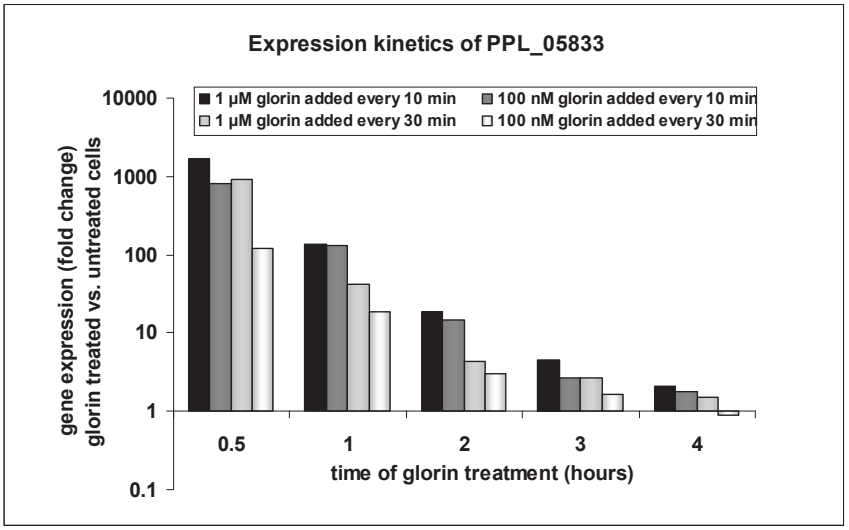


Figure 34: Glorin-induced changes in expression of PPL_05833 in response to varying concentrations and periods of exposure to glorin. *P. pallidum* PN500 were developed in shaking cultures for 5 hours in the presence or absence of exogenous glorin. In glorin treated cultures, beginning 1 hour after starvation, either 1 µM or 100 nM final concentration of glorin was added at 10 or 30-minute intervals. Cell samples were collected after 0.5-, 1-, 2-, 3- and 4-hours of glorin treatment for total RNA extraction to analyse gene expression. Cells were generally harvested 30 minutes after a pulse of glorin was applied. Samples were collected from untreated cells at the same time points. As a control, expression of house keeping gene *gpdA* was examined. Relative expression of model genes PPL_05833 was determined by real-time RT-PCR. Fold changes are shown. All data were compared to untreated *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represent higher expression of the gene in glorin treated cells than in untreated cells. Values < 1 show that expression of gene is lower in glorin treated

cells than in untreated cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted. 4 hours of glorin treatment represented total 5 hours of starvation.

PPL_12271 is another glorin-induced gene that encodes putative ErkB protein. This gene exhibited kinetics similar to PPL_05833 in this experiment. PPL_12271 was rapidly induced by glorin within 30-minute of glorin treatment, followed by a gradual decline, such that after 4 hours, expression of PPL_12271 droped to near background levels even in the presence of frequent glorin pulsing. These results confirm RNA-seq data that also indicated a rapid turn over of PPL_12271 (Figure 26 & 35). Analogous to PPL_05833, the product of this gene seems to be required for short period during aggregation and may play important role in intracellular cell signalling. PPL_12271 was induced to almost comparable levels in response to 1 µM and 100 nM glorin pulsed at 10- minute intervals. 1 µM glorin added every 30-minute showed similar response as displayed by pulses delivered more frequently. 100 nM glorin pulsed at 30-minute intervals could induce PPL_12271 only at very low levels, pointing that induction of this gene may require high levels of glorin (Figure 35).

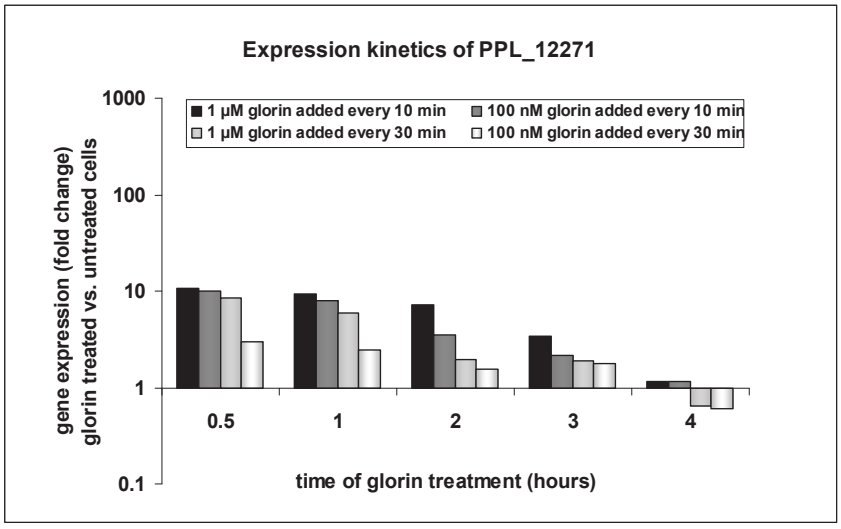


Figure 35: Glorin-induced changes in expression of PPL_12271 in response to varying concentrations and periods of exposure to glorin. *P. pallidum* PN500 were developed in shaking cultures for 5 hours in the presence or absence of exogenous glorin. In glorin treated cultures, beginning 1 hour after starvation, either 1 µM or 100 nM final concentration of glorin was added at 10 or 30-minute intervals. Cell samples were collected after 0.5-, 1-, 2-, 3- and 4-hours of glorin treatment for total RNA extraction to analyse gene expression. Cells were generally harvested 30 minutes after a pulse of glorin was applied. Samples were collected from untreated cells at the same time points. As a control, expression of house keeping gene *gpdA* was examined. Relative expression of model genes PPL_12271 was determined by real-time RT-PCR. Fold changes are shown. All data were compared to untreated *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represent higher expression of the gene in glorin treated cells than in untreated cells. Values < 1 show that expression of gene is lower in glorin treated

cells than in untreated cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted. 4 hours of glorin treatment represented total 5 hours of starvation.

An important comment to the data obtained with PPL_05833 and PPL_12271 is related to transient expression of these genes. RNA-seq data indicated that expression of many genes that were differentially up-regulated more than 3-fold was declined below the set threshold (3-fold expression) 1 hour later (Table 3). It was suspected that glorin levels may need to be elevated continually for the expression of this set of genes to be maintained. Therefore, when expression of some of these genes (Figure 34 & 35) was analyzed under frequent pulsing coniditons, it was found that even frequent exposure to glorin caused decline in gene expression. This provides evidence that transient changes in gene expression induced by glorin are an innate property of starving *P. pallidum* PN500 cells.

PPL_07908 is an example of a glorin-repressed gene. As indicated in figure 28, PPL_07908 is highly induced by starvation during the early hours of starvation but treatment with glorin rapidly suppresses expression of this gene. Expression data of PPL_07908 presented in Figure 36 confirmed the findings of RNA-seq analyses by showing that expression of this gene is strongly repressed by glorin during the early hours of development.

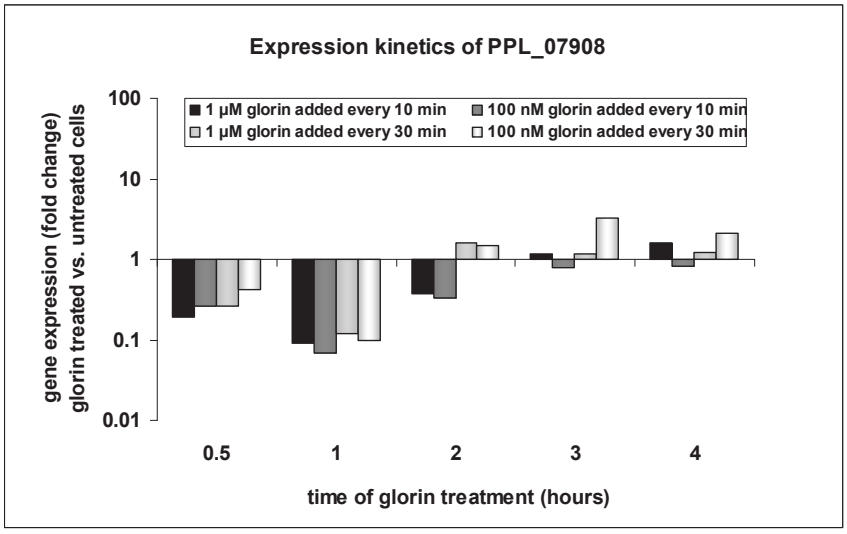


Figure 36: Glorin-induced changes in expression of PPL_07908 in response to varying concentrations and periods of exposure to glorin. *P. pallidum* PN500 were developed in shaking cultures for 5 hours in the presence or absence of exogenous glorin. In glorin treated cultures, beginning 1 hour after starvation, either 1 µM or 100 nM final concentration of glorin was added at 10 or 30-minute intervals. Cell samples were collected after 0.5-, 1-, 2-, 3- and 4-hours

of glorin treatment for total RNA extraction to analyse gene expression. Cells were generally harvested 30 minutes after a pulse of glorin was applied. Samples were collected from untreated cells at the same time points. As a control, expression of house keeping gene *gpdA* was examined. Relative expression of model genes PPL_07908 was determined by real-time RT-PCR. Fold changes are shown. All data were compared to untreated *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represent higher expression of the gene in glorin treated cells than in untreated cells. Values < 1 show that expression of gene is lower in glorin treated cells than in untreated cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted. 4 hours of glorin treatment represented total 5 hours of starvation.

When cells were treated with 1 µM or 100 nM glorin (at 10 or 30-minutes intervals) for 1 hour, expression of PPL_07908 declined to insignificant levels. As shown in Figure 36, expression of PPL_07908 remained downregulated in response to frequent pulsing of glorin (1 µM or 100 nM glorin pulsed at 10-minute intervals) for 4 hours, as indicated by gene expression levels after 2-, 3- and 4- hours of glorin treatment in Figure 36. However, when cells were pulsed with 100 nM glorin at 30-minute intervals, low level expression of PPL_07908 was detected after 3 and 4 hours of treatment. These data may indicate that expression of PPL_07908 is repressed by high concentrations of glorin, whereas low level expression of this gene is recovered when intensity of glorin pulses is reduced.

Alternatively, these results demonstrate that to maintain down-regulation of PP_07908 for longer period, comparatively higher levels of glorin are required at shorter intervals. This gene may be an immediate early developmental gene induced by starvation and suppression of this gene by glorin indicates that products of this gene may not be necessary during aggregation.

3.8.3 Temporal expression pattern of glorin responsive genes during development in shaken suspensions

After determining relationships among glorin pulse concentration, pulsing interval and related gene expression changes, next the developmental kinetics of glorin responsive genes were determined in *P. pallidum* PN500 amoebae developed in shaken cultures over longer periods of time (i.e. more than 5 hours) to get an insight into how gene expression patterns develop when cells are starved in buffer suspensions. For this experiment, two different concentrations of glorin, i.e. 1 µM & 100 nM were chosen and time-course effects of frequent glorin pulsing were examined on gene expression patterns of selected glorin-induced genes during the first 8 hours of development. 8 hour duration was selected to cover the complete period of early development in suspension cultures; however, chemotactic aggregation of cells is prevented under these conditions.

P. pallidum PN500 cells were grown in association with bacteria, harvested in the late vegetative stage, washed and resuspended in phosphate buffer at a concentration of $2x10^7$cells/ml. A pellet of $2x10^7$cells was collected for total RNA isolation that represented 'growing cells control' for subsequent gene expression analyses. Cells in suspension cultures were pre-starved for 1 hour under slow shaking conditions (i.e. 100 rpm) at 22^{o}C to initiate development. After 1 hour of starvation, cell suspension was divided into 3 parts. One culture served as 'untreated control' and did not receive glorin treatment. Second culture was treated with 1 µM glorin every 10-minutes, whereas in third culture 100 nM glorin was added at 10-minute intervals. These cultures were developed under shaking conditions at 22^{o}C for 8 additional hours. Samples (each containing $2x10^7$ cells) were collected from glorin-treated cultures starting at 0.5 hour of glorin treatment until total 8 hours for RNA extraction. Samples were collected from untreated cell cultures at the same time points. Cell samples were generally collected 30- minutes after a pulse of glorin was delivered. cDNA was syntheiszed and gene expression analyses were carried out using quantitative RT-PCR. Relative gene expressions of selected glorin-induced genes PPL_09347, PPL_05354, PPL_05833, PPL_12271 and a glorin repressed gene PPL_07908 were determined and fold changes were calculated compared to expression in growing cells. 8 hours of glorin treatment corresponded to 9 hours of starvation (1 hour pre-starvation + 8 hours glorin treatment).

3.8.3.1 Class I: Genes stably induced by glorin in shaking cultures

3.8.3.1.1 Expression profile of PPL_09347

When expression kinetics of model gene PPL_09347 were analyzed in cells developed in the absence of glorin treatment, only basal level expression of this gene could be detected at 1.5 hour of development. Expression was then maintained at low levels for the next 4 hours (Figure 37).

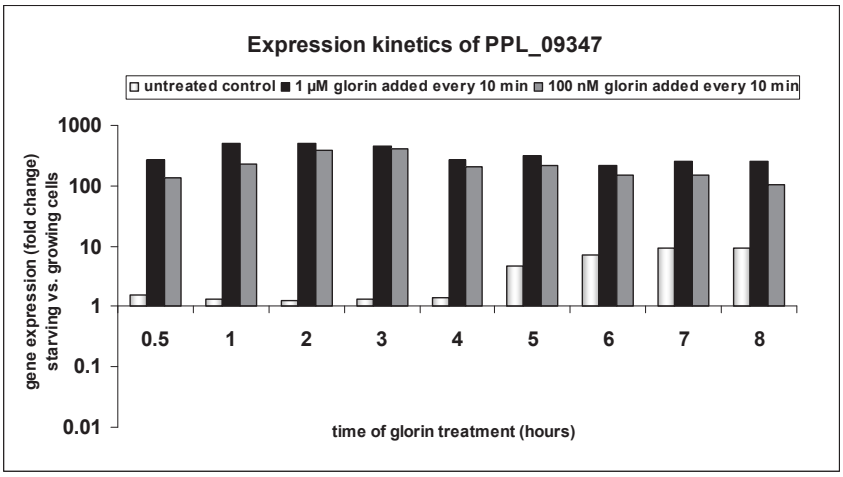


Figure 37: Time course of glorin effects on PPL_09347 expression. *P. pallidum* PN500 were developed in shaking buffer suspensions for 8 hours in the presence or absence of exogenous glorin. In glorin treated cultures, beginning 1 hour after starvation, either 1 µM or 100 nM final concentration of glorin was added at 10-minute intervals. Cell samples were collected at indicated time points after glorin treatment for total RNA extraction to analyse gene expression. Cells were generally harvested 30 minutes after a pulse of glorin was applied. Samples were collected from untreated cells at the same time points. As a control, expression of house keeping gene *gpdA* was examined. Relative expression of model genes PPL_09347 was determined by real-time RT-PCR. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of gene is lower in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

Levels of PPL_09347 mRNA started to rise in untreated cells after 5 hours of development and continued to increase gradually for the next hours of observation. These time points may correspond to aggregation time during normal development on agar. Only basal level expression of PPL_09347 during the first 4 hours of development may indicate that product of this gene is not needed in the first few hours of starvation.

When cells were pulsed with glorin every 10 minutes, mRNA of PPL_09347 accumulated rapidly within 30-minutes (0.5 hour of glorin treatment; Figure 37) and 265- and 136.32-fold induction was detected in response to 1 µM and 100 nM glorin pulses, respectively, delivered at 10-minute intervals. In the presence of glorin stimulus, high-level expression of PPL_09347 was maintained in the next 8 hours of observation. Gene induction in response to both concentrations of glorin used (1 µM & 100 nM) was comparable at all time points. These data indicate that PPL_09347 is constantly induced by glorin to high levels. These results also show that within a few hours of development in suspensions,

cells may establish endogenous glorin signalling (referred to the expression kinetics of PPL_09347 in untreated control shown in Figure 37) and start to secrete glorin themselves; however, these signalling may not reach the same level as in glorin-pulsed cultures (Figure 37).

3.8.3.1.2 Expression profile of PPL_05354

When gene expression data collected from *P. pallidum* PN500 cells starved in shaking suspensions for 8 hours was analyzed, it was noticed that in the absence of glorin pulses, PPL_05354 exhibited nearly negligible expression during the first 6 hours of development in shaking suspensions. A slight increase in the expression was observed starting at 7 hours of starvation, while 3.03-, 5.5-, and 10.7-fold expression levels were detected at 7-, 8- and 9-hour of development, respectively (Figure 38). These results suggest that PPL_05354 is gradually induced in the course of development.

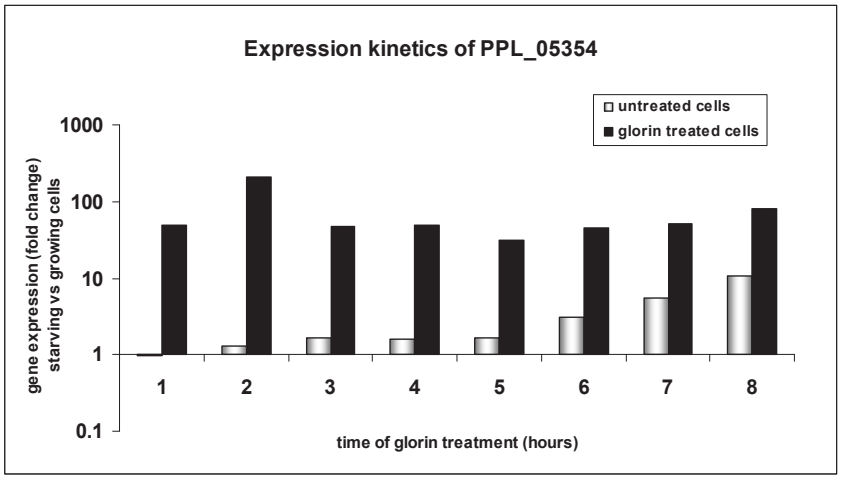


Figure 38: Time course of glorin effects on PPL_05354 expression. *P. pallidum* PN500 were developed in shaking buffer suspensions for 8 hours in the presence or absence of exogenous glorin. In glorin treated cultures, beginning 1 hour after starvation, 1 µM final concentration of glorin was added at 30-minute intervals and cell samples were collected at indicated time points after glorin treatment for total RNA extraction to analyse gene expression. Cells were generally harvested 30 minutes after a pulse of glorin was applied. Samples were collected from untreated cells at the same time points. As a control, expression of house keeping gene *gpdA* was examined. Relative expression of model gene PPL_05354 was determined by real-time RT-PCR. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells and fold change was set to 1 where values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of gene is lower in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

When cells were treated with 1 µM glorin at 30-minute intervals, PPL_05354 was induced rapidly, such that 48.5-fold expression (compared to growing cells) could be detected within 1 hour of glorin treatment (Figure 38). After stimulation of cells with glorin for 2 hours, this gene was further up-regulated to 210.83-fold followed by a decline in expression to 47.17-, 48.5- and 31.12-fold at 3-, 4-, and 5-hours of glorin treatment, respectively (Figure 38). A slight increase in expression was noticed at 6- and 7-hours of glorin treatment, whereas 82.13-fold expression was detected in cells treated with glorin for 8 hours (Figure 38). Effects of pulsing 100 nM glorin were not detected. These data indicate that PPL_05354 is rapidly induced by glorin to high levels and gene expression in response to exogenous glorin was stable over 8 hours of observation.

3.8.3.2 Class II: Genes induced by starvation, whereas exogenous glorin pulses resulted in their precocious expression

3.8.3.2.1 Expression profile of PPL_05833

Real-time RT-PCR data analyses showed that PPL_05833 was expressed to insignificant levels during the first 3 hours of development in shaking culture (Table 3; Figure 27). In response to starvation in suspension culture, a slight increase in the expression of PPL_05833 was observed starting at 4 hours of development, whereas 10.41-fold expression was detected at 7 hours of starvation (Figure 39). Expression was then maintained at approximately the same levels in the next hours of development (Figure 39). It may indicate that products of this gene are not required during the early hours of starvation. RNA-seq analyses predicted that PPL_05833 is a gene that is transiently induced by glorin (Figure 27). This experiment verified that PPL_05833 is rapidly induced by glorin to high levels within 30 minutes, follwed by a steep decline, such that after 2 hours of glorin treatment only a low level expression could be detected (Figure 39).

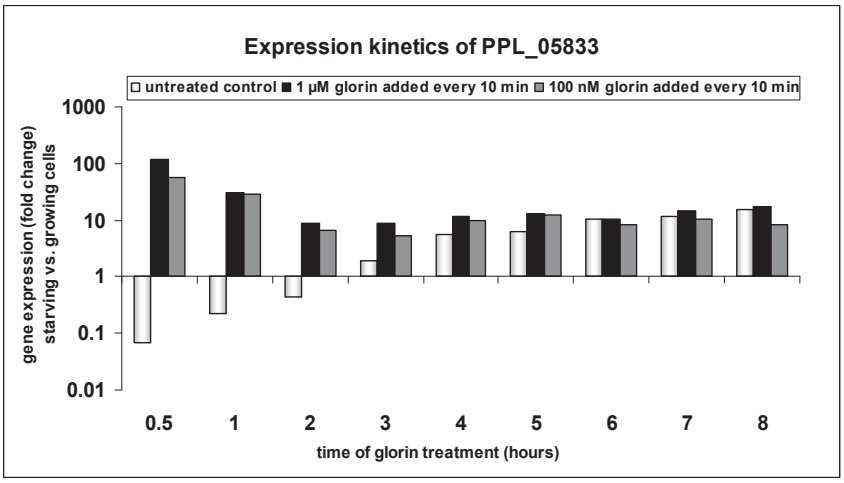


Figure 39: Time course of glorin effects on PPL_05833 expression. *P. pallidum* PN500 were developed in shaking buffer suspensions for 8 hours in the presence or absence of exogenous glorin. In glorin treated cultures, beginning 1 hour after starvation, either 1 µM or 100 nM final concentration of glorin was added at 10-minute intervals. Cell samples were collected at indicated time points after glorin treatment for total RNA extraction to analyse gene expression. Cells were generally harvested 30 minutes after a pulse of glorin was applied. Samples were collected from untreated cells at the same time points. As a control, expression of house keeping gene *gpdA* was examined. Relative expression of model genes PPL_05833 was determined by real-time RT-PCR. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of gene is lower in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

Effects of pulsing 1 µM or 100 nM glorin were comparable. These data validate observations made in previous section of this study (Section 3.8.2; Figure 34) that PPL_05833 is induced in response to glorin only for a short period, afterwards expression is retained at basal levels; supporting the suggestion that products of this gene may not be needed for long time during aggregation.

3.8.3.2.2 Expression profile of PPL_12271

PPL_12271 (*erkB*) is a gene that displayed kinetics similar to PPL_05833. In the absence of glorin pulses only very few transcripts of this gene were detected during the first 4 hours of development in suspension cultures. A low level expression was noticed at 5 hour of starvation that was maintained almost at the same levels for the next hours of development (Figure 40).

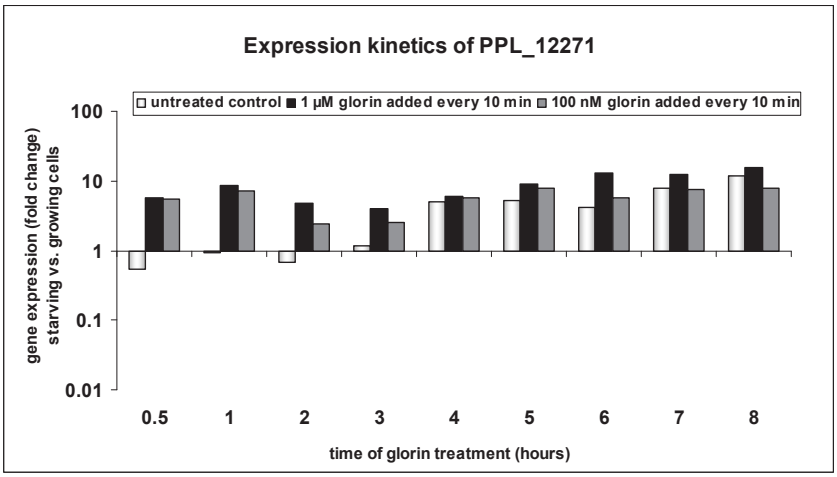


Figure 40: Time course of glorin effects on PPL_12271 expression. *P. pallidum* PN500 were developed in shaking buffer suspensions for 8 hours in the presence or absence of exogenous glorin. In glorin treated cultures, beginning 1 hour after starvation, either 1 µM or 100 nM final concentration of glorin was added at 10-minute intervals. Cell samples were collected at indicated time points after glorin treatment for total RNA extraction to analyse gene expression. Cells were generally harvested 30 minutes after a pulse of glorin was applied. Samples were collected from untreated cells at the same time points. As a control, expression of house keeping gene *gpdA* was examined. Relative expression of model genes PPL_12271 was determined by real-time RT-PCR. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of gene is lower in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

As shown in figure 40, PPL_12271 was induced within 30-minutes in the presence of 1 µM or 100 nM pulses of glorin. Expression declined briefly at 2 and 3 hours of glorin treatment. A slight increase in expression of this gene was detected at 4 hour of stimulation with glorin that was maintained at low levels in the next hours. 1 µM and 100 nM concentrations of glorin used for pulsing were almost equally effective. PPL_12271 exhibits rapid changes in expression during the early hours of starvation in response to glorin treatment (Figure 26 & 40); indicating that activity of this gene may be required only for short time during aggregation.

3.8.3.3 Class III: Gene induced by starvation, whereas glorin pulses repressed their expression during the early hours of development

3.8.3.3.1 Expression profile of PPL_07908

PPL_07908 is a starvation induced early gene that was shown to be repressed by glorin treatment earlier in this study (Section 3.6.2.1.4 Figure 28; Figure 36).

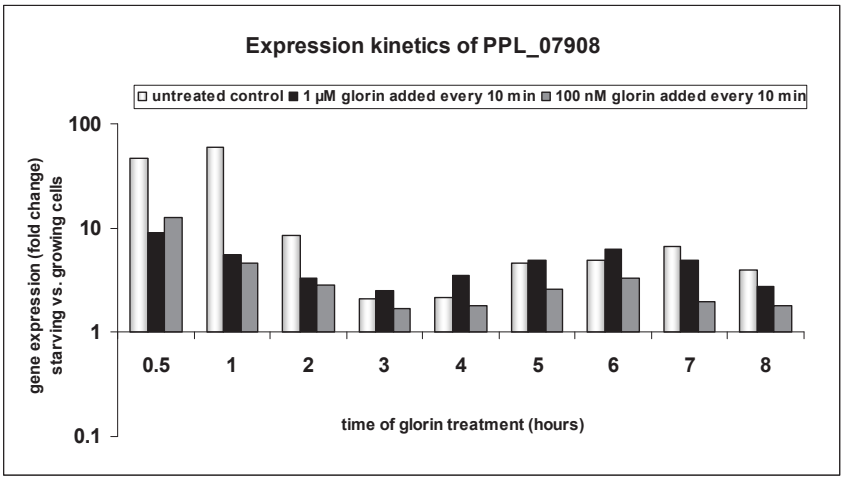


Figure 41: Time course of glorin effects on PPL_07908 expression. *P. pallidum* PN500 were developed in shaking buffer suspensions for 8 hours in the presence or absence of exogenous glorin. In glorin treated cultures, beginning 1 hour after starvation, either 1 µM or 100 nM final concentration of glorin was added at 10-minute intervals. Cell samples were collected at indicated time points after glorin treatment for total RNA extraction to analyse gene expression. Cells were generally harvested 30 minutes after a pulse of glorin was applied. Samples were collected from untreated cells at the same time points. As a control, expression of house keeping gene *gpdA* was examined. Relative expression of model genes PPL_07908 was determined by real-time RT-PCR. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of gene is lower in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

As shown in figure 41, this gene was rapidly induced in response to starvation in cells developed in shaking suspensions. High expression levels of this gene were noticed for first 2 hours of starvation followed by a steep decline, such that at 4 and 5 hours of development (in the absence of glorin), only basal level expression was detected. A slight increase in expression was observed beginning at 6 hours of starvation that was then maintained at the same level in the next hours. These data add to the suggestion

that PPL_07908 is an early developmental gene required during the initial hours of starvation only.

Pulses of glorin delivered at 10-minutes intervals led to a rapid decline in the expression of PPL_07908, such that only basal level of expression was detected at 3 and 4 hours of glorin treatment. Afterwards (at 5 to 8 hours of glorin treatment) this gene was expressed at low levels; almost similar expression levels were detected both in glorin-treated and untreated cells at these time points (Figure 41). 1 µM and 100 nM glorin pulses were comparably effective during the first 3 hours of glorin treatment. Responses to both concentrations of glorin were very slightly different at later stages.

3.8.3.4 Class IV: Genes repressed by starvation, whereas glorin pulses induce their expression in shaking cultures

3.8.3.4.1 Expression profile of PPL_12248

As shown in Figure 29, RNA-seq data indicated that PPL_12248 is a gene that is repressed by starvation but glorin pulses induce its expression. In experiment presented in Figure 42, when *P. pallidum* PN500 amoebae were starved in shaking suspensions for 8 hours, continuous down-regulation of PPL_12248 was noticed over the whole time period confirming the results of RNA-seq analyses that this gene is negatively regulated by starvation (Figure 29 & Figure 42).

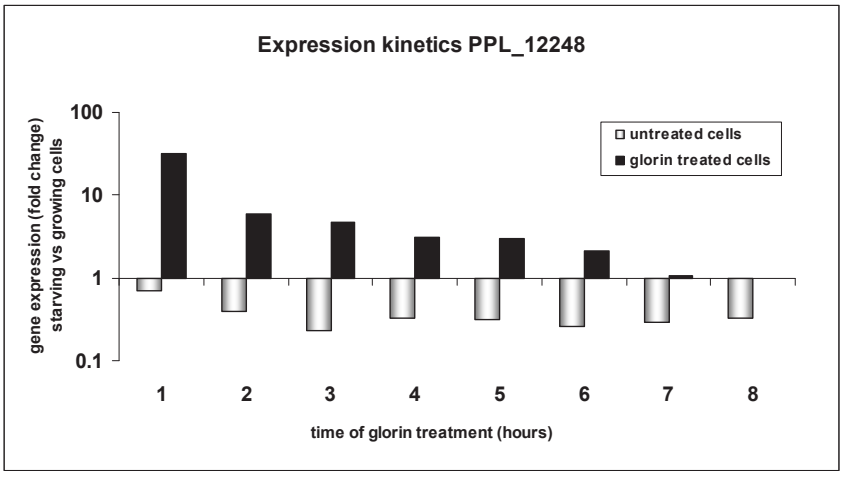


Figure 42: Time course of glorin effects on PPL_12248 expression. *P. pallidum* PN500 were developed in shaking buffer suspensions for 8 hours in the presence or absence of exogenous

glorin. In glorin treated cultures, beginning 1 hour after starvation, 1 µM final concentration of glorin was added at 30-minute intervals and cell samples were collected at indicated time points after glorin treatment for total RNA extraction to analyse gene expression. Cells were generally harvested 30 minutes after a pulse of glorin was applied. Samples were collected from untreated cells at the same time points. As a control, expression of house keeping gene *gpdA* was examined. Relative expression of model gene PPL_12248 was determined by real-time RT-PCR. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells and fold change was set to 1 where values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of gene is lower in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

In comparison, when cells were pulsed with 1 µM glorin at 30-minute intervals, transient increase in expression was noticed. Within one hour of treatment with glorin, expression of this gene increased by 31.55-fold. Expression decreased gradually thereafter, such that 5.93-, 4.65-, 3.13-, 3.03-, 2.12-, 1.06- and 1-fold expression was detected at 2-, 3-, 4-, 5-, 6-, 7- & 8-hours of glorin treatment, respectively (Figure 42). Effects of pulsing 100 nM glorin were not studied. These data indicate that expression of PPL_12248 is highly induced by glorin for short time period, illustrating a possible short-term activity of this gene during aggregation.

3.8.3.4.2 Expression profile of PPL_12249

PPL_12249 is a gene that exhibits expression kinetics similar to PPL_12248. Upon starvation of *P. pallidum* PN500 amoebae in shaking cultures for 8 hours, a constant down-regulation of PPL_12249 gene expression was observed (Figure 43) demonstrating that this gene is developmentally repressed and may represent a ‘growing stage gene’. While analyzing absolute expression levels of this gene (Table 3), it could be noticed that PPL_12249 transcripts were present in growing cells, however, at the onset of starvation number of transcripts declined confirming that development leads to down-regulation of this gene.

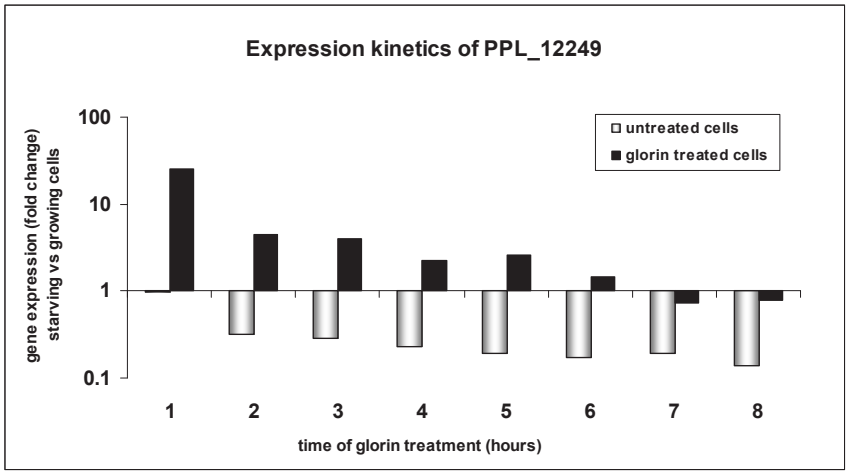


Figure 43: Time course of glorin effects on PPL_12249 expression. *P. pallidum* PN500 were developed in shaking buffer suspensions in the presence or absence of exogenous glorin. In glorin treated cultures, beginning 1 hour after starvation, 1 µM final concentration of glorin was added at 30-minute intervals for an additional 8 hours and cell samples were collected at indicated time points for total RNA extraction to analyse gene expression. Cells were generally harvested 30 minutes after a pulse of glorin was applied. Samples were collected from untreated cells at the same time points. As a control, expression of house keeping gene *gpdA* was examined. Relative expression of model gene PPL_12249 was determined by real-time RT-PCR. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells and fold change was set to 1 where values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of gene is lower in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

P. pallidum PN500 cells exhibited a high-level expression of PPL_12249 after 1 hour of glorin treatment (1µM glorin delivered at 30-minute intervals) and 25.28-fold upregulation could be observed. Gene expression declined sharply at later time points but was still detectable until 6 hours of glorin treatment; expression was down-regulated afterwards. 4.5-, 3.97-, 2.28-, 2.56-, and 1.46-fold gene expression was detected at 2-, 3-, 4-, 5- and 6-hours of glorin treatment (Figure 43). Effects of pulsing 100 nM glorin were not tested. Transient induction of PPL_12249 to high levels by exogenous glorin indicates that this gene may play a short-term but rather significant role in glorin signaling during aggregation. As shown in Figure 43, continuous supression of PPL_12249 by starvation over the course of observation (i.e. 8 hours) indicates that this gene may be a 'vegetative stage gene'. However, glorin treatment transiently induced expression of PPL_12249 to high levels; pointing to the possibility that momentary activity of this gene may be required during aggregation also though this gene may function mainly during growth phase of cells.

Overall, these data suggest that suspension cultures provide an efficient setting to study glorin-induced changes in gene expression patterns in starving *P. pallidum* PN500 amoebae and cells successfully complete early developmental changes.

3.8.4 Developmental time course of gene expression in cells starved on non-nutrient agar plates

P. pallidum PN500 development is a highly synchronous process and is accompanied by a series of coordinated morphological and physiological changes. It starts with the transition from growth to aggregation that is induced by starvation. The major morphological change occurs when the amoebae begin to aggregate in response to communication with a diffusible chemoattractant that may be glorin as suggested by experiments performed in this study (described under Section 3.4.1.1 & 3.6.2). The activity of cells and chemotaxis to acrasin mediate the aggregation of groups of cells into loose aggregates, followed by continuous strong streaming movements towards aggregation centres leading to the formation of tipped aggregates (Figure 44).

After studying developmental regulation of glorin-responsive genes in *P. pallidum* PN500 cells starved in shaking suspensions, the temporal expression patterns of selected glorin-induced genes were examined in cells developing on agar surface because development of amoebae on solid substrata is closer to the natural conditions than developing in a shaking culture. With this approach, the aim was to analyze distinct changes in gene expression patterns associated with morphological transitions and to determine which developmental event correspond to maximum increase in expression of glorin-regulated genes.

P. pallidum PN500 cells were grown in association with bacteria on 1/5 SM agar plates. When plates were clear, cells were collected, washed and resuspended in phosphate buffer at concentration of $2x10^7$cells/ml. A pellet of $2x10^7$ vegetative stage cells was collected for total RNA extraction and served as ‘growing cells control’ for gene expression studies. Cells were spread as monolayers on non-nutrient agar plates at a density of $8X10^5$cells/cm^2 for development. Cells ($2x10^7$/sample) were harvested at 9 different stages of early development including initial 1-4 hours of starvation (the pre-aggregation stages) and distinct morphological states indicated by loose aggregates (5 hour), aggregates (6.5 hour), streaming (8.5 hour), late aggregates (10 hour) and mounds (13.5 hour) in Figure 44.

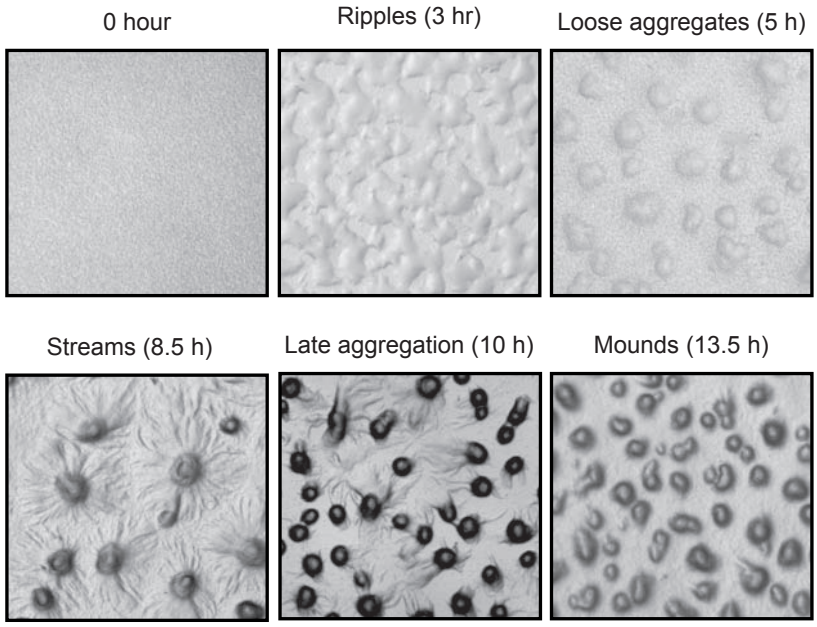


Figure 44: Prominent morphological states during growth-to-aggregation transition of starving *P. pallidum* PN500 amoebae. A top view of cells developing on non-nutrient agar is shown. No multicellular structures were visible at 0 hour. Ripples (3 hours), loose aggregates (5 hours), streams (8.5 hours), late aggregation (10 hours), and mounds (13.5 hours) are shown.

Using this strategy, the goal was to encompass all steps of growth-to-aggregation transition for analyzing temporal expression of glorin responsive genes. Total RNA was extracted from samples that were collected beginning at the onset of starvation until formation of mounds and temporal gene expression profiles were analyzed for selected glorin-regulated genes.

Genes including PPL_09347, PPL_05354, PPL_11763, PPL_00912, PPL_03541 and PPL_06644 were found to be developmentally regulated exhibiting maximum changes in gene expression between 4 and 8 hours of development; time points which correspond to the transition from unicellular to multicellular organization and are accompanied by most dramatic morphological changes in development of *P. pallidum* PN500. Genes such as PPL_12271 and PPL_ 05833 were expressed at low levels during the early hours of development, whereas PPL_12248 and PPL_03784 were expressed at basal levels approximately at all 9 developmental states that were monitored in this study. It has been suggested that individual group of genes expressed in the same manner may represent specialized patterns in the course of development (Loomis & Shaulsky 2011). It is also proposed that a gene whose expression increases by at least four fold during

development probably encodes a protein that is likely to provide selective advantage under one or another condition (Loomis & Shaulsky 2011).

The results are interpreted on gene-by-gene basis in the following sections.

3.8.4.1 Class I: Aggregation stage specific genes

3.8.4.1.1 Developmental kinetics of PPL_09347

RNA-seq data indicated that bacterially grown vegetative-stage *P. pallidum* PN500 cells accumulate a low level of PPL_09347 mRNA (Table 3), however, as shown in Figure 45, development on agar led to a gradual increase of the mRNA level which reached a peak at 10 hour of development that corresponds to late aggregation stage (Figure 44). Within first 3 hours of development, only basal level expression could be detected. Expression levels increased thereafter, such that at 6.5 hours of development when aggregation was in process, 12.89-fold expression (compared to growing cells) could be detected that continued to increase during streaming. Maxmium level expression i.e. 30.27-fold was noticed with the formation of late aggregates, whereas expression levels declined to 22.16-fold when mounds were observed.

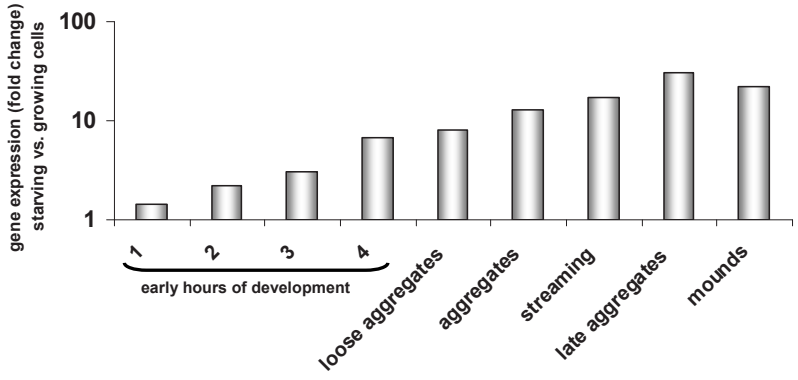


Figure 45: Developmental regulation of PPL_09347. *P. pallidum* PN500 cells grown in association with bacteria were harvested in the late vegetative stage, washed, and resuspended in phosphate buffer. Cells were then starved on phosphate agar plates at a density of $8X10^5$cells/cm^2. Cell samples were collected at indicated developmental stages for total RNA extraction. Relative expression of model gene PPL_09347 was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represented higher expression of the gene in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

These data indicate that under natural conditions, the expression level of PPL_09347 starts to increase when cells shift from pre-aggregation to aggregation phase and continues to rise during streaming until formation of tight aggregates. These data confirm RNA-seq findings that also detected only basal level expression during the early hours of development (Figure 25). Under shaking culture conditions, in the absence of glorin, mRNA level of PPL_09347 started to rise at 6 hour of development and 9.18-fold expression was detected by 8 hour of development that was maintained at the same level in the next hour (Figure 37). No more increase in gene expression could be noticed under shaking conditions, indicating that normal level expression of this gene (as indicated in Figure 45) may require sufficient cell-cell contacts. Moreover, high level induction of this gene by glorin under shaking culture conditions suggested that this gene is developmentally regulated by glorin. On agar surface, continuous up-regulation of PPL_09347 during the entire aggregation process further validates the indication that cells might have fully established glorin signalling during aggregation phase leading to maximum level induction of PPL_09347.

3.8.4.1.2 Developmental kinetics of PPL_03541

In *P. pallidum* PN500 amoebae developed on phosphate agar plates, basal-level expression of PPL_03541 was detected during the first 3 hours of starvation. Moderate increase in expression of this gene was detected at 4- and 5-hour of development (Figure 46). Expression of this gene continued to rise, reached a peak in actively aggregating cells and was then maintained at moderate levels in cells forming streams and tight aggregates (Figure 46). Thus, in the early phase of development of *P. pallidum*, there seems to be a close temporal correlation between accumulation of mRNA of PPL_03541 and the appearance of aggregates. A slight decline in expression was noticed thereafter (Figure 46). Expression levels detected in cells starved for 1-, 2- and 3-hours were 1.5-, 2.43- and 2.81-fold higher, respectively, than in growing cells. At 4- and 5-hour of development 5.36- and 7.95-fold increase in expression was observed, respectively (Figure 46). Compared to growing cells, this gene was 11.95-fold upregulated at 6.5 hour of development. During streaming and late aggregation stages of development 8.31- and 8.15-fold increase in expression was detected. PPL_03541 was 6.16-fold upregulated when cells had formed mounds (Figure 46).

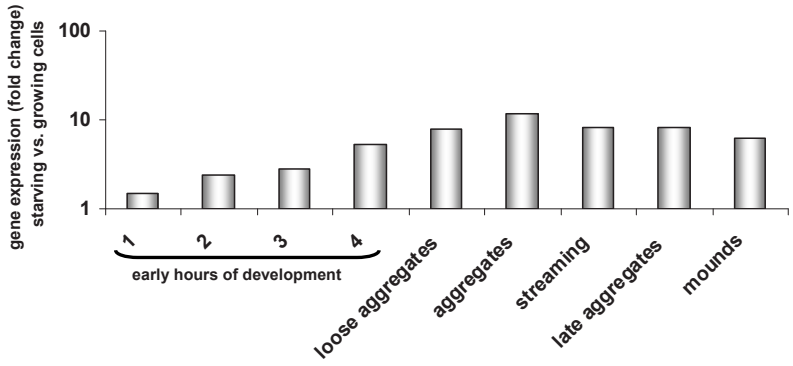


Figure 46: Developmental regulation of PPL_03541. *P. pallidum* PN500 cells grown in association with bacteria were harvested in the late vegetative stage, washed, and resuspended in phosphate buffer. Cells were then starved on phosphate agar plates at a density of $8X10^5$cells/cm^2. Cell samples were collected at indicated developmental stages for total RNA extraction. Relative expression of model gene PPL_03541 was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represented higher expression of the gene in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

Absolute expression data obtained in RNA-seq experiment showed that mRNA of PPL_03541 was barely detectable in growing cells (Table 3). Negligible expression of this gene was detected in amoebae developed for 2- or 3-hours in shaking cultures (Table 3). However, when cells were treated with exogenous glorin for 1 hour, 9.44-fold higher expression was detected than in growing cells, whereas expression of PPL_03541 was downregulated after cells were treated with glorin for 2 hour demonstrating that PPL_03541 is developmentally induced by glorin for relatively short periods (Figure 26). Rapid decline of glorin-induced moderate increase in transcript levels of this gene in shaking cultures may result from instability of mRNA under these conditions. Maximal expression of PPL_03541 during aggregation may indicate that PPL_03541 plays a specific role during aggregation of *P. pallidum* PN500 amoebae.

3.8.4.1.3 Developmental kinetics of PPL_06644

When *P. pallidum* PN500 amoebae were developed on non-nutrient agar and expression profile of PPL_06644 was analyzed, it was noticed that this gene is developmentally induced. In the first 2 hours of starvation, only basal level expression was detected (1.1- & 1.29-fold, respectively; Figure 47). With progression of starvation, expression of this gene increased gradually and reached a peak (23-fold higher than in

growing cells; Figure 47) during the late aggregation stage. When cells have formed mounds, expression of PPL_06644 declined a little indicating that activity of this gene is required mainly during pre-aggregation and aggregation stages of development.

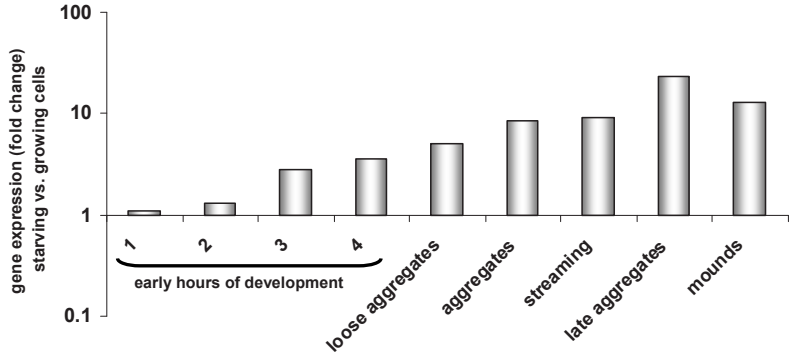


Figure 47: Developmental regulation of PPL_06644. *P. pallidum* PN500 cells grown in association with bacteria were harvested in the late vegetative stage, washed, and resuspended in phosphate buffer. Cells were then starved on phosphate agar plates at a density of $8X10^5$cells/cm^2. Cell samples were collected at indicated developmental stages for total RNA extraction. Relative expression of model gene PPL_06644 was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represented higher expression of the gene in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

RNA-seq data indicated that PPL_06644 is moderately induced by glorin (Table 3). If aggregating *P. pallidum* PN500 amoebae secrete glorin, then an induction of PPL_06644 is anticipated during aggregation phase of development and data presented in Figure 47 supports this assumption by indicating that PPL_06644 is upregulated throughout the aggregation process. These features show that PPL_06644 is an aggregation stage gene.

3.8.4.2 Class II: Genes exhibiting characteristics of both 'early genes' and 'aggregation-stage genes'

3.8.4.2.1 Developmental kinetics of PPL_11763

Previously, RNA-seq data indicated that PPL_11763 is expressed in growing cells to very low levels (Table 3 presenting absolute expression data). Also, it was noticed that expression of PPL_11763 is gradually induced by starvation, whereas glorin treatment

leads to transient upregulation of this gene to high levels (Figure 26). When cells were washed free of bacteria and allowed to undergo normal development on phosphate agar plates, transcripts of PPL_11763 were detectable by 1 hr of development, expression levels increased gradually, while maximal expression was detected at late aggregation stage until formation of mounds. Rapid accumulation of this gene to 2.56-, 7.31-, 8.22- and 17.26-fold at 1-, 2-, 3- and 4-hours of development (time points corresponding to pre-aggrgeation period), respectively indicates that PPL_11763 is an early gene (Figure 48).

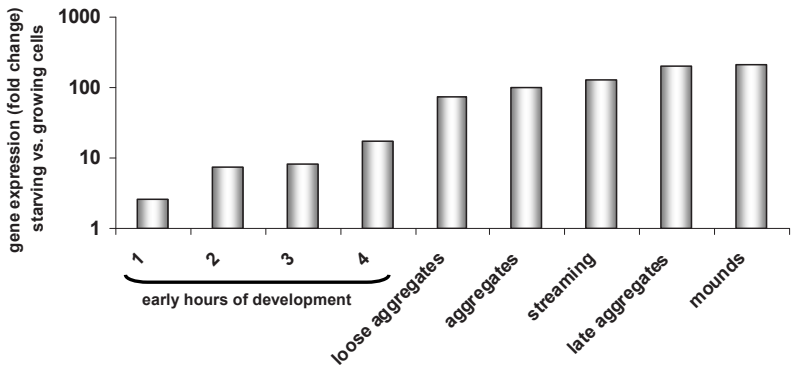


Figure 48: Developmental regulation of PPL_11763. *P. pallidum* PN500 cells grown in association with bacteria were harvested in the late vegetative stage, washed, and resuspended in phosphate buffer. Cells were then starved on phosphate agar plates at a density of $8X10^5$cells/cm^2. Cell samples were collected at indicated developmental stages for total RNA extraction. Relative expression of model gene PPL_11763 was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represented higher expression of the gene in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

Further increase in expression levels of this gene was noticed during the whole process of aggregation. At early aggregation stage 74.54-fold expression of PPL_11763 was detected that increased to 129.78-fold in actively streaming cells (Figure 48). Expression of this gene extends beyond the aggregation stage and a high level of transcript persists in the postaggregation stages; 200.85- and 210.83-fold higher expression of PPL_11763 was dectected at late aggregation and mound stage, repectively (Figure 48). These data support the indication provided by RNA-seq analyses that PPL_11763 displays characteristics of both early gene and aggregation-stage gene. Overall, these results and RNA-seq data may indicate that PPL_11763 is regulated both by starvation and glorin. Rapid turn over of glorin-induced moderate-level increase in the amounts of PPL_11763

transcripts in shaking cultures may result from instability of mRNA of this gene under these conditions.

3.8.4.2.2 Developmental kinetics of PPL_05354

While analyzing expression pattern of PPL_05354 at various times of development, it was found that this gene was 4.19-fold upregulated after 1 hour of starvation compared to growing cells (Figure 49). PPL_05354 was further expressed moderately by 10.33- and 12.99-fold at 2- and 3-hours of development suggesting that this gene is induced by starvation during the early hours of development (Figure 49).

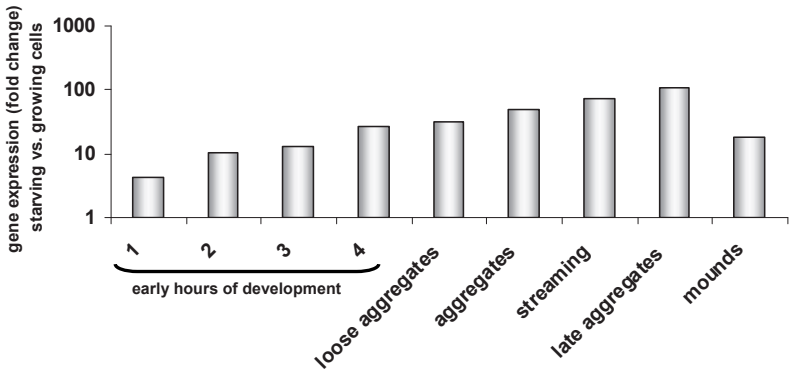


Figure 49: Developmental regulation of PPL_05354. *P. pallidum* PN500 cells grown in association with bacteria were harvested in the late vegetative stage, washed, and resuspended in phosphate buffer. Cells were then starved on phosphate agar plates at a density of $8X10^5$cells/cm^2. Cell samples were collected at indicated developmental stages for total RNA extraction. Relative expression of model gene PPL_05354 was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represented higher expression of the gene in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

Expression levels increased to 33.22-fold at 5 hour of development; time corresponding to early aggregation stage. A continuous rise in expression was observed at next hours of development, such that 71.25- and 106.15-fold upregulation could be detected at streaming and late aggregation stages (Figure 49). High level expression of PPL_05354 during the developmental hours corresponding to aggregation illustrate that this gene may be additionally modulated by glorin secreted by aggregating cells. Expression of PPL_05354 then declined at mound stage indicating that activity of this gene is moderately required during pre-aggregation stages, optimally needed in the course of aggregation and once aggregates have formed, effects of PPL_05354 decline. These

data suggest that PPL_05354 is a gene that exhibits features of both early gene and aggregation-stage gene. These results are in agreement with RNA-seq analysis that showed the presence of adequate number of transcripts of PPL_05354 in amoebae that were developed for 2 and 3 hour (Table 3) and demonstrated that this gene is additionally strongly induced by glorin (Figure 25). Moreover, RNA-seq data favours the assumption that activity of PPL_05354 might be required during the growth phase also because sufficient transcripts of this gene were detected in growing cells (Table 3).

3.8.4.2.3 Developmental kinetics of PPL_00912

Accumulation of PPL_00912 was determined during the first 14 hours of development of P. pallidum PN500 amoebae on non-nutrient agar that indicated a progressive increase in expression levels of this gene (Figure 50). At 1 and 2 hours of development 1.49- and 4.62-fold expression was noticed, respectively. Transcript levels increased to 7.48- and 10.5-fold in cells starved for 3 and 4 hours of development, respectively (Figure 50).

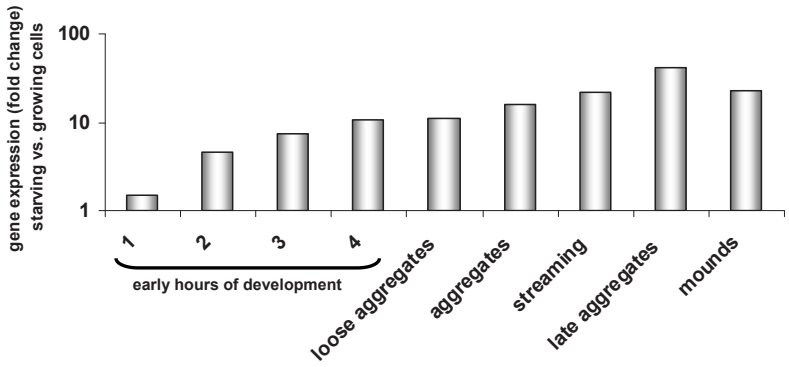


Figure 50: Developmental regulation of PPL_00912. *P. pallidum* PN500 cells grown in association with bacteria were harvested in the late vegetative stage, washed, and resuspended in phosphate buffer. Cells were then starved on phosphate agar plates at a density of $8X10^5$cells/cm^2. Cell samples were collected at indicated developmental stages for total RNA extraction. Relative expression of model gene PPL_00912 was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represented higher expression of the gene in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

Expression of this gene increased step-by-step during early aggregation and streaming stages, peaking at late aggregation stage where 41.18-fold expression was detected, whereas gene expression declined to relatively low-levels at mound stage (Figure 50). These data further confirm the findings of RNA-seq analyses suggesting that

PPL_00912 is a starvation-induced gene, while expression of this gene is further enhanced by glorin signalling (Figure 27). Therefore, in parallel with PPL_11763 and PPL_05354, PPL_00912 also possesses characteristics of both early developmental gene and aggregation-stage gene.

3.8.4.3 Class III: Genes nearly similarly expressed during the early stages of development

3.8.4.3.1 Developmental kinetics of PPL_12271 *(erkB)*

PPL_12271 is a gene with distinct kinetics (Figure 51). RNA-seq analyses indicated that this gene is transiently up-regulated by glorin (Figure 26). Analysing absolute expression data of PPL_12271 showed that sufficient transcripts of this gene were present in growing cells and cells starved for 1 hour, while transcript number declined slightly at 2 hours of development (Table 3). When *P. pallidum* PN500 cells were developed on agar surface, 4.11-fold higher expression was detected in cells starved for 1 hour than in growing cells (Figure 51).

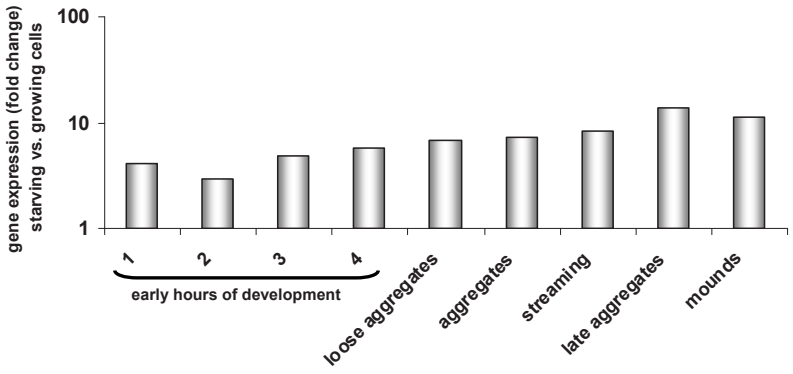


Figure 51: Developmental regulation of PPL_12271 (*erkB*). *P. pallidum* PN500 cells grown in association with bacteria were harvested in the late vegetative stage, washed, and resuspended in phosphate buffer. Cells were then starved on phosphate agar plates at a density of $8X10^5$cells/cm^2. Cell samples were collected at indicated developmental stages for total RNA extraction. Relative expression of model gene PPL_12271 was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represented higher expression of the gene in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

Expression decreased to 2.98-fold at 2 hour of development. Expression levels started to rise afterwards, such that 6.88- and 8.28-fold expression was detected in cells forming

early aggregates and streams, respectively. Expression reached a peak level (13.73-fold) by 10 hours of development when tight aggregates of cells appeared (Figure 51). At mound stage, 11.15-fold expression was noticed. These data indicate that PPL_12271 is expressed in cells at moderate levels during all the stages of early development, whereas expression levels are slightly enhanced during aggregation supporting moderate level induction of this gene by glorin. Sufficient expression of PPL_12271 in growing cells and at different stages of development may demonstrate important role played by this gene during growth and development.

3.8.4.4 Class IV: Genes expressed at basal level during the early stages of development

3.8.4.4.1 Developmental kinetics of PPL_03784

When expression profile of PPL_03784 was analyzed in cells developing on non-nutrient agar plates, a low level expression of this gene was noticed during the first 4 hours of starvation. A small increase in expression of PPL_03784 was detected between 5 and 10 hour of development (Figure 52). At about 5 hour of development on agar surface, formation of early aggregates is observed followed by aggregation streams at 8.5 hour (Figure 44). Thus, a slight increase in expression of PPL_03784 within 5 to 10 hours of development might be connected to aggregation phase of cells. Transcripts levels of PPL_03784 appeared to peak at late aggregation stage when 7.11-fold higher expression was detected than in growing cells. Expression declined slightly at the mound stage (Figure 52). Gene expression data of PPL_03784 obtained in RNA-seq experiment indicated that PPL_03784 is a developmentally induced gene and treatment of starving cells with exogenous glorin further increases expression of this gene (Figure 27). Analyses of absolute expression data showed that this gene is insignificantly expressed in growing cells, whereas starvation led to an increase in number of transcripts of this gene at 2 and 3 hours of development (Table 3); results that correlate with the findings depicted in Figure 52. Moreover, RNA-seq data indicated that PPL_03784 is transiently up-regulated by glorin and 20-fold increase in expression was detected in response to treatment with exogenous glorin for 1 hour that declined to 7.96-fold after cells were treated with glorin for total 2 hours in shaking cultures (Table 3; Figure 27).

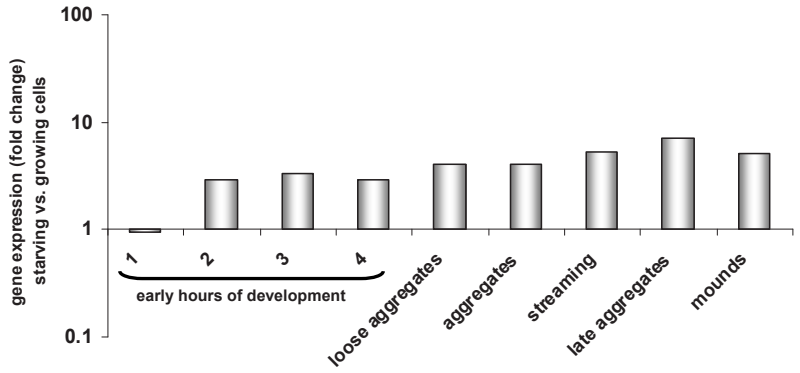


Figure 52: Developmental regulation of PPL_03784. *P. pallidum* PN500 cells grown in association with bacteria were harvested in the late vegetative stage, washed, and resuspended in phosphate buffer. Cells were then starved on phosphate agar plates at a density of $8X10^5$cells/cm^2. Cell samples were collected at indicated developmental stages for total RNA extraction. Relative expression of model gene PPL_03784 was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change in growing cells was defined as 1 where values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of gene is lower in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

However, cells developed on agar surface exhibited maximal expression of PPL_03784 at late aggregation stage when 7.11-fold increase in expression was detected (Figure 52). The difference between induction of expression of PPL_03784 in cells developed in shaking cultures in the presence of glorin and those starved on agar surface can be explained by assuming that aggregating amoebae of *P. pallidum* PN500 excrete glorin, nevertheless, transient changes in the expression of PPL_03784 in response to secreted glorin were not much prominent. This phenomenon may point to the difference in the behaviour of cells in shaking cultures and on agar surface. Another explanation could be that rapid and transient nature of gene expression changes did not allow for detection of short-lived, high-level upregulation of PPL_03784 by secreted glorin within the time points when cell samples were collected for gene expression analysis. These data suggest that PPL_03784 may play critical but short-term role during aggregation of *P. pallidum* PN500 amoebae.

3.8.4.4.2 Developmental kinetics of PPL_05833

PPL_05833 is a gene that exhibits expression profile almost similar to that of PPL_03784. When amoebae were starved on phosphate agar plates for 1 hour, PPL_05833 displayed low level of expression and compared to growing cells, only 1.23-

fold increase in expression was detected (Figure 53). Expression of this gene increased to 3.05-, 3.58-, 4.14-fold at 2-, 3- and 4-hour of development, respectively (Figure 53). During aggregation, a slight increase in expression of PPL_05833 was detected. Messenger RNA complementary to PPL_05833 peaked at 10 hour of development and 7.51-fold higher expression of this gene was noted in cells forming tight aggregates than in growing cells (Figure 53). At mound stage, expression declined slightly. Moderate level expression of this gene at almost all stages of early development indicates that PPL_05833 may perform important biological role during the initial stages of development of *P. pallidum* PN500 amoebae.

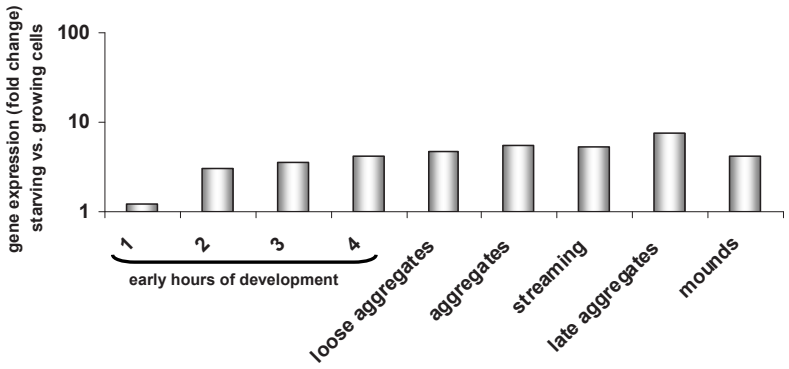


Figure 53: Developmental regulation of PPL_05833. *P. pallidum* PN500 cells grown in association with bacteria were harvested in the late vegetative stage, washed, and resuspended in phosphate buffer. Cells were then starved on phosphate agar plates at a density of $8X10^5$cells/cm^2. Cell samples were collected at indicated developmental stages for total RNA extraction. Relative expression of model gene PPL_05833 was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represented higher expression of the gene in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

In RNA-seq experiment, when absolute expression data of PPL_05833 was analyzed (Table 3), it was found that this gene is insignificantly expressed in vegetative cells. RNA-seq analysis further showed that when cells are developed in shaking cultures, PPL_05833 is induced by starvation, while stimulation of starving amoebae with exogenous glorin further increases expression of this gene transiently. 133.2-fold increase in expression of PPL_05833 was induced in response to stimulation with glorin for 1 hour, while only 25.75-fold increase in expression could be detected after 2 hours of treatment with glorin (Figure 27 A). When data obtained with cells developed on non-nutrient agar are compared with results from cells starved in shaking cultures for the same time period, it is noticed that expression of PPL_05833 is developmentally

regulated in both cases (Figure 39 & 53). On the other hand, high-level but transient induction of PPL_05833 was detected when cells developing in shaking suspensions were treated with glorin (Figure 27 & 39). Speculating that aggregating amoebae of *P. pallidum* PN500 secrete glorin, a high-level expression of PPL_05833 is expected in cells undergoing aggregation on agar surface. However, data obtained from cells developed on non-nutrient agar do not provide such an indication and PPL_05833 is only moderately expressed during aggregation (Figure 53). These differences can be attributed to the rapid and transient induction of PPL_05833 by glorin. It is quite possible that this gene is induced to high-levels in *P. pallidum* PN500 cells developing on agar surface in response to glorin secreted by aggregating cells but such a change might be so fast and short-term that could not be detected in any of the cell samples collected at those specific stages of development shown in Figure 44. However, shaking cultures are not comparable to the natural conditions of development of amoebae and 1 µM glorin used to stimulate cells at 10- or 30-minute intervals is not representative of natural pulsing frequency of glorin secreted by starving amoebae during aggregation. Therefore, different sensitivities of cells developing in shaking cultures or on agar surface may also explain differences in gene induction levels in response to externally added or secreted glorin.

3.8.4.4.3 Developmental kinetics of PPL_12248

Analyses of gene expression data obtained from cells developed on phosphate agar plates showed that PPL_12248 is expressed at basal levels throughout the early development (Figure 54). At 1-, 2-, 3- and 4-hour of development, 1.99-, 2.18-, 1.56-, and 1.37-fold higher expression (than in growing cells) was detected, respectively (Figure 54). At the onset of aggregation expression of this gene increased to 2.25 fold, was maintained at 2.44-fold during streaming and reduced to 1.09-fold at mound stage (Figure 54).

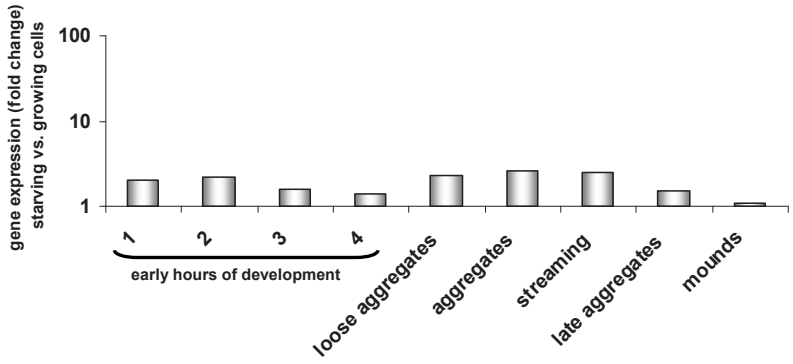


Figure 54: Developmental regulation of PPL_12248. *P. pallidum* PN500 cells grown in association with bacteria were harvested in the late vegetative stage, washed, and resuspended in phosphate buffer. Cells were then starved on phosphate agar plates at a density of $8X10^5$cells/cm^2. Cell samples were collected at indicated developmental stages for total RNA extraction. Relative expression of model gene PPL_12248 was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represented higher expression of the gene in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

RNA-seq data showed that some transcripts of PPL_12248 were present in vegetative cells that declined at the onset of starvation (Table 3), indicating that PPL_12248 may be a 'growth-stage-specific gene' that is repressed by starvation. However, it was shown that stimulation of suspension developed *P. pallidum* PN500 cells with exogenous glorin led to significant level induction of expression of PPL_12248 (Figure 29), such that after 1 hour of exposure to glorin 35.62-fold increase in gene expression could be noticed compared to expression in growing cells, whereas only 7-fold expression was detected in cells treated with glorin for 2 hours; illustrating rapid and transient induction of PPL_12248 in response to glorin (Figure 29). When *P. pallidum* PN500 cells were developed for 8 hours in shaking cultures in the absence of glorin, a continuous down-regulation of this gene was detected, however, transient and high-level induction of PPL_12248 was noticed in the presence of exogenous glorin (Figure 42). When amoebae are developed on non-nutrient agar, the pattern of PPL_12248 gene expression is somewhat different from that observed in cells developed in shaking cultures. Though PPL_12248 is constantly down-regulated under shaking culture conditions, yet it exhibits a very low level expression in cells developing on agar surface (Figure 42 & 54); indicating that cell-cell contacts may be required for low-level expression of this gene. PPL_12248 shows characteristics of vegetative-stage gene but is rapidly induced by glorin. This distinct kinetics of expression of PPL_12248 may demonstrate that glorin is detected by growing cells also and signal is transduced to

downstream effectors that leads to gene expression changes during growth phase. Other justification could be that PPL_12248 is rapidly induced to moderate- or high-levels in response to glorin secreted by cells aggregating on agar surface but induction might be short-lived and increased levels of expression decline back to basal level in a short period of time, therefore, could not be detected within the sampling time points as shown in Figure 54. Transient induction of PPL_12248 by glorin may explain short-term activity of this gene during aggregation of *P. pallidum* PN500 amoebae.

3.8.5 Developmental regulation of selective GPCRs

GPCRs are seven-transmembrane proteins that transduce extracellular signals inside the cell by activating heterotrimeric G-proteins that couple receptors to effector proteins inside the cell to trigger physiological responses (Sucgang *et al.* 2011). Previous studies indicated that glorin acts by binding to cell surface G-protein coupled receptors (De Wit *et al.* 1988). It was demonstrated that aggregating amoebae of *P. violaceum* possess cell surface receptors for the detection of the extracellular glorin (De Wit *et al.* 1988). However, it was shown that vegetatively growing amoebae of *P. violaceum* also expose adequate number of glorin receptors on their cell surface that increases slightly during aggregation (De Wit *et al.* 1988). In *D. discoideum* expression of cAR1 is induced by pulses of extracellular cAMP during aggregation (Mu *et al.* 1998; Firtel 1995; Loomis 1996; Louis *et al.* 1993; Saxe *et al.* 1991a, b; Klein *et al.* 1988, 1987; Kimmel 1987). RNA-seq analyses conducted in this study indicated that in *P. pallidum* PN500 a number of GPCRs were positively regulated by glorin to varying degrees (Table 4). It was suspected that gene encoding glorin receptor(s) may be among the GPCR encoding genes differentially regulated by external glorin; similar to the induction of aggregation stage cAR1 by extracellular pulses of cAMP in *D. discoideum*. It was therefore interesting to analyze developmental kinetics of selected glorin-regulated GPCR encoding genes during the early hours of development of *P. pallidum* PN500 cells. These experiments were designed to recognize GPCR genes that were expressed in growing cells and their expression was slightly enhanced during the process of aggregation. GPCR gene(s) exhibiting such kinetics might represent candidate glorin receptor gene(s).

P. pallidum PN500 cells grown in association with bacteria were harvested in the late vegetative stage, washed, and resuspended in phosphate buffer. Cells were then starved on phosphate agar plates at a density of $8x10^5$cells/cm^2. Cell samples were collected at 9

different developmental stages for total RNA extraction (Figure 44) and developmental kinetics of selected glorin-induced GPCR encoding genes were analyzed.

3.8.5.1 Developmental kinetics of GPCR genes induced by glorin

Developmentally regulated GPCR genes were divided into 3 major classes based on their respective kinetics.

3.8.5.1.1 Class I: GPCR encoding genes expressed in growing amoebae, whose expression levels are enhanced upon starvation

RNA-seq data demonstrated that PPL_00902 and PPL_05727 were two distinct GPCR genes whose transcripts were moderately expressed in vegetative cells, whereas stimulation of cells with glorin for 1 hour led to 2.74- and 4.15-fold increase in expression of PPL_00902 and PPL_05727, respectively (Table 3). When *P. pallidum* PN500 amoebae were developed on agar, starvation moderately enhanced expression of PPL_00902, such that at all stages of development analyzed in this experiment, PPL_00902 was almost similarly regulated (Figure 55). Expression of PPL_05727 was also enhanced in response to starvation but kinetics was different compared to PPL_00902. PPL_05727 exhibited low-level increase in expression during the first 4 hours of starvation (Figure 55). At the onset of aggregation, expression of this gene further increased and was maintained at high-levels during the whole process of aggregation (Figure 55). PPL_00902 and PPL_05727 represent ideal candidate genes that may encode glorin receptors because both of these genes are expressed moderately in growing cells and their expression is further enhanced with progression of starvation.

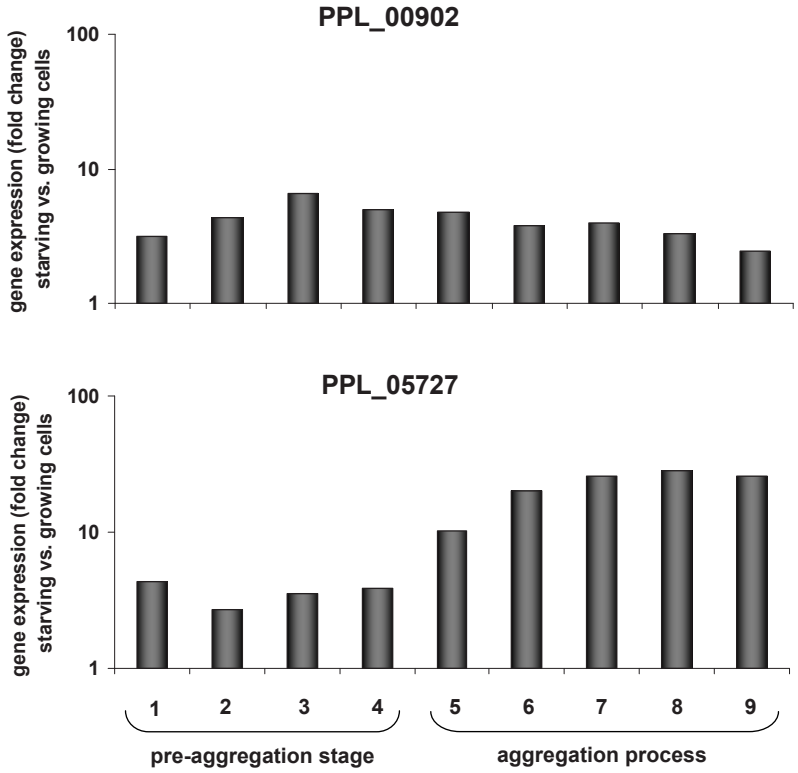


Figure 55: Developmental regulation of GPCR genes PPL_00902 & PPL_05727. *P. pallidum* PN500 cells were starved on non-nutrient agar. Cell samples were collected at 9 different stages of early development including initial 1-4 hours of starvation (the pre-aggregation stage) and distinct morphological states indicated by number 5 (loose aggregates), 6 (aggregates), 7 (streaming), 8 (late aggregates) and 9 (mounds) for total RNA extraction. Relative expression of PPL_00902 and PPL_05727 was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represented higher expression of the gene in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

3.8.5.1.2 Class II: Starvation induced GPCRs encoding genes whose expression is augmented during aggregation

Certain GPCR genes, of which PPL_ 04108 and PPL_00855 are obvious examples, exhibited negligible expression in growing cells (referred to absolute gene expression data presented in Table 3). Starvation caused a gradual increase in expression of PPL_04108 and a maximal level expression was observed during aggregation that decreased to low-levels after tight aggregates have formed (Figure 56). PPL_00855 exhibited comparatively different kinetics. This gene was moderately expressed at 1 hour of starvation, followed by a gradual decrease in expression until 4 hour of

development on agar (Figure 56). A moderate increase in the expression of PPL_00855 was then noticed throughout the process of aggregation (Figure 56). These genes are selectively induced in response to starvation and may play vital roles during aggregation.

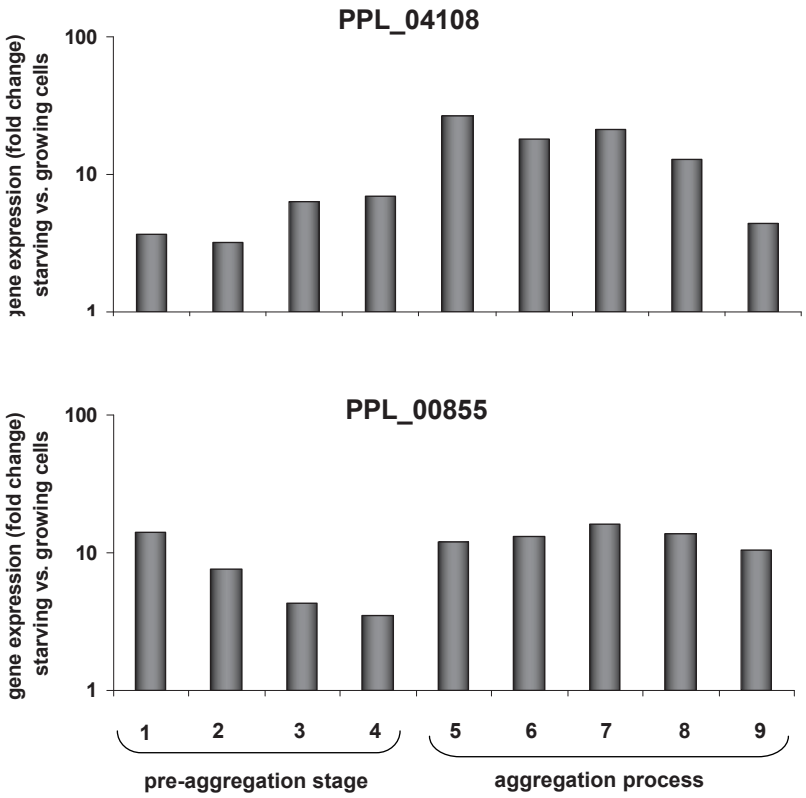


Figure 56: Developmental regulation of GPCR genes PPL_04108 & PPL_00855. *P. pallidum* PN500 cells were starved on non-nutrient agar. Cell samples were collected at 9 different stages of early development including initial 1-4 hours of starvation (the pre-aggregation stage) and distinct morphological states indicated by number 5 (loose aggregates), 6 (aggregates), 7 (streaming), 8 (late aggregates) and 9 (mounds) for total RNA extraction. Relative expression of PPL_04108 and PPL_00855 was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represented higher expression of the gene in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

3.8.5.1.3 Class III: GPCR encoding genes expressed specifically during aggregation

Some GPCR genes, such as PPL_08454, PPL_08455, and PPL_03564 were characteristically induced at the onset of aggregation and their expression was maintained at moderate levels during aggregation (Figure 57). Expression of these genes

was insignificant in growing cells (Table 3). Products of these genes may play essential roles during aggregation.

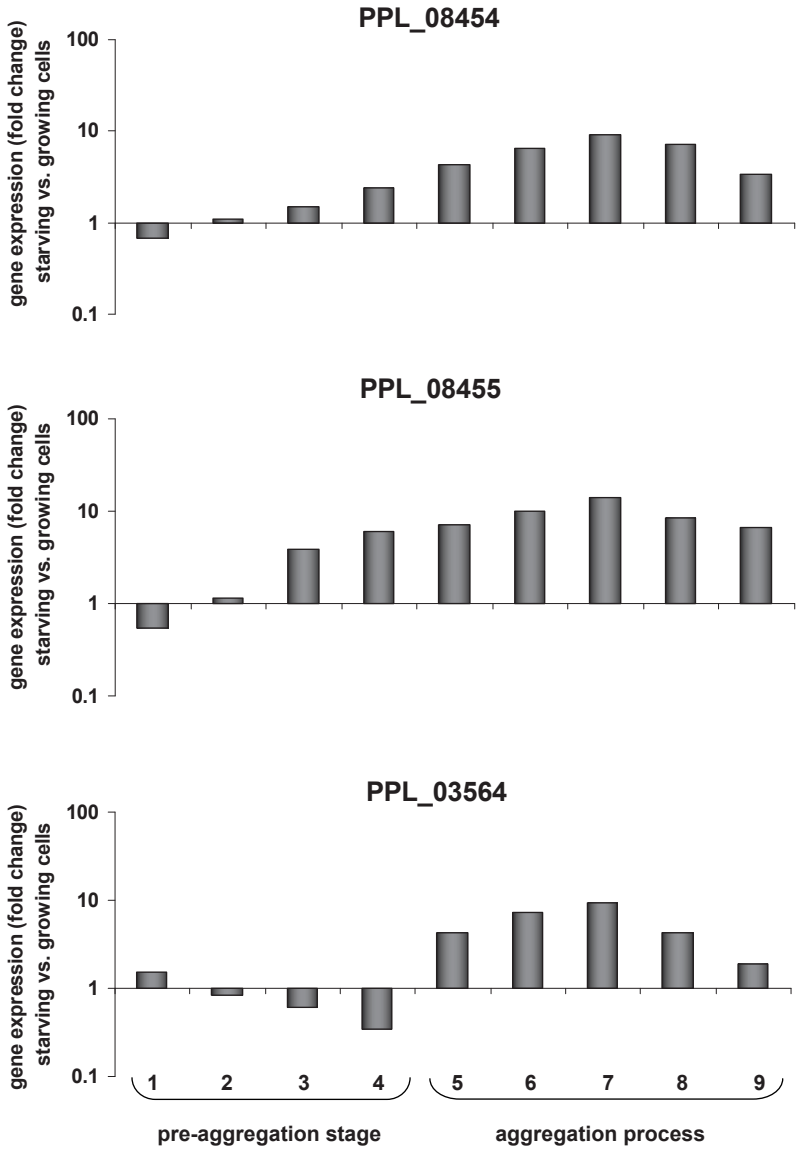


Figure 57: Developmental regulation of GPCR genes PPL_08454, PPL_08455 & PPL_03564. *P. pallidum* PN500 cells were starved on non-nutrient agar. Cell samples were collected at 9 different stages of early development including initial 1-4 hours of starvation (the pre-aggregation stage) and distinct morphological states indicated by number 5 (loose aggregates), 6 (aggregates), 7 (streaming), 8 (late aggregates) and 9 (mounds) for total RNA extraction. Relative expression of PPL_08454, PPL_08455 and PPL_03564 was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells. Fold change was set to 1 where values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of gene is lower in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

Expression kinetics of selected glorin-regulated genes in *P. pallidum* PN500 cells

Gene Name	Developed in shaken suspensions for 8 hours		Developed on non-nutrient agar plates until formation of mounds
	Effects of starvation only	Effects of glorin treatment	
PPL_09347	Only slightly induced by starvation during the first 4 hours of development, moderately induced afterwards	Stably induced by glorin to high-levels	Expression increased gradually during the early hours of development, highly expressed during aggregation
PPL_06644	Insignificant expression during the first few hours of development	Transiently induced by glorin, followed by a rapid decline in expression	Expression increased gradually during the early hours of development, highly expressed during aggregation
PPL_05357	Only slightly induced by starvation during the first 4 hours of development, moderately induced afterwards	Stably induced by glorin to high-levels	Expression increased gradually during the early hours of development, highly expressed during aggregation
PPL_11763	Expressed at basal levels during the first 3 hours of development	Transiently induced by glorin to high levels, followed by a rapid decline in expression	Expression increased gradually during the early hours of development, highly expressed during aggregation
PPL_12271	Expressed at basal levels during the first 3 hours of development, gradual increase of expression to moderate levels is observed afterwards	Transiently induced by glorin, followed by a rapid decline in expression	Basal level expression for the first 2 hours. Expressed at low levels during the later stages of development investigated with a slight increase during aggrgeation
PPL_03541	Expressed at basal levels during the first 3 hours of development	Transiently induced by glorin to moderate levels, followed by a rapid decline in expression levels	Expression increased gradually during the early hours of development, moderately expressed during aggregation
PPL_05833	Gradually induced by starvation to moderate levels	Glorin treatment further enhanced its expression rapidly, followed by a decline in expression.	Basal level expression in the first hour of starvation. Expressed at low levels during the later stages of development investigated (starting from 2nd hour of starvation until mound formation)

PPL_03784	Induced by starvation	Glorin treatment further enhanced its expression rapidly, followed by a gradual decline in expression	Basal level expression in the first hour of starvation. Expressed at low levels during the later stages of development investigated (starting from 2nd hour of starvation until mound formation)
PPL_00912	Induced by starvation	Glorin treatment further enhanced its expression, follwed by a gradual decline in expression	Expression increased gradually during the early hours of development, highly expressed during aggregation
PPL_07908	Highly induced by starvation during the first 2-3 hours of starvation followed by a rapid decline at 4-5 hours of development. Afterwards, expression increased gradually to moderate levels	Glorin treatment repressed its expression during the first 2-3 hours of development	Not detected
PPL_05702	Induced by starvation	Glorin treatment repressed its expression	Not detected
PPL_05195	Induced by starvation	Glorin treatment repressed its expression	Not detected
PPL_12248	Repressed by starvation	Stimulation of cells with glorin induced its expression to high levels for the first hour of treatment, followed by a gradual decrease in expression	Expressed at basal levels during all the stages of development investigated (Figure 44)
PPL_12249	Repressed by starvation	Stimulation of cells with glorin induced its expression to high levels for the first hour of treatment, followed by a gradual decrease in expression	Expressed at basal levels during all the stages of development investigated
PPL_00902	Expressed in growing amoebae, induced by starvation	Expression enhanced by glorin	Expressed in growing amoebae, expression enhanced to moderate levels during the early stages of development
PPL_05727	Expressed in growing amoebae, induced by starvation to moderate levels	Expression enhanced by glorin to moderate levels	Expressed in growing amoebae, expression enhanced gradually and reached a maximum during aggregation

PPL_04108	Induced by starvation to moderate levels	Expression enhanced by glorin to moderate levels	Moderately expressed during the early hours of development, expression enhanced gradually and reached a maximum during aggregation
PPL_00855	Induced by starvation to low levels	Expression enhanced by glorin to low levels	Moderately expressed during the early hours of development, expression enhanced gradually and reached a maximum during aggregation
PPL_08454	Induced by starvation to low levels	Rapidly induced by glorin to moderate levels, followed by a rapid decline	Expression enhanced gradually during the early hours of development and reached a maximum during aggregation
PPL_08455	Induced by starvation to low levels	Rapidly induced by glorin to moderate levels, followed by a rapid decline	Expression enhanced gradually during the early hours of development and reached a maximum during aggregation
PPL_03564	Induced by starvation to low levels	Rapidly induced by glorin to moderate levels, followed by a rapid decline	Expression enhanced gradually during the early hours of development and reached a maximum during aggregation

Table 5: Summarized expression kinetics of chosen glorin-regulated genes in *P. pallidum* PN500 cells developed in shaking cultures (in the presence or absence of glorin) and on non-nutrient agar plates.

3.9 Glorin signalling function independent of the cAMP signaling system

Cyclic AMP signalling is used to organize the post-aggregative stages of development throughout the *Dictyostelids* phylogeny (Schaap 2011a; Kawabe *et al.* 2009; Alvarez-Curto *et al.* 2005). However, in younger group 4 species, secreted cAMP acts both as a chemoattractant to mediate aggregation of starving amoebae and as a developmental signal to trigger pre-spore differentiation (Schaap 2011a; Sucgang *et al.* 2011; Schaap 2007). It has been shown that cAMP is secreted by starving *P. pallidum* amoebae (Konijn *et al.* 1968) and a *pdsA* gene (*phosphodiesterase A*) showing low affinity for cAMP is expressed in *P. pallidum* PN500 cells during growth, aggregation and post-aggregation stages (Kawabe *et al.* 2012). However, the 'acrasin of aggregation' in this species is not cAMP (Shaffer 1962; Jones & Robertson 1976; Shimomura *et al.* 1982), therefore, it is assumed that the low-affinity phosphodiesterase may not be efficacious to generate steep cAMP gradients during aggregation (Kawabe *et al.* 2012). After aggregation has completed, there is a major reorganization of the control of development in *P. pallidum* where intercellular communication within the multicellular slug exploits cAMP as chemotactic agent for the organization of post-aggregative morphogenesis. Therefore, it was interesting to study whether there is any cross-talk between glorin and cAMP, i.e. chemoattractant coordinating post-aggregation stages of *P. pallidum* development.

3.9.1 Expression patterns of components of cAMP signalling system are not affected by glorin stimulus

First, effects of stimulating starving *P. pallidum* PN500 cells with glorin were studied on expression patterns of chosen cAMP signalling system genes.

P. pallidum PN500 amoebae were harvested from bacterial growth plates, washed free of bacteria and resuspended in phosphate buffer at cell density $2x10^7$cells/ml. A pellet of $2x10^7$cells served as 'vegetative-stage growing cells control' for subsequent gene expression analysis. Cell suspension was then equally divided into two flasks. One suspension culture of *P. pallidum* PN500 amoebae was prestarved for 1 hour, and then treated with periodic additions of 1 µM glorin at 30-min intervals for up to 2 additional hours to determine the influence of externally added glorin on the expression pattern of selected genes known to be involved in cAMP signalling (Figure 58). Other culture was

maintained in the absence of added glorin for the time period. Cells samples were taken from glorin treated and untreated cultures at 2- and 3-hours of development for total RNA extraction to analyse changes in gene expression. Expression patterns of adenylyl cyclase A (*acaA2*; PPL_01657), phosphodiesterase A (*pdsA*; PPL_10234), cAMP-dependent protein kinase C (*pkaC*; PPL_05049), and a homolog of cAMP receptor gene named *tasB* are shown in Figure 58. It was noted that stimulation of starving *P. pallidum* PN500 amoebae with glorin has no obvious influence on the expression patterns of all tested genes involved in cAMP signalling demonstrating that glorin signalling may not augument post-aggregation cAMP signalling.

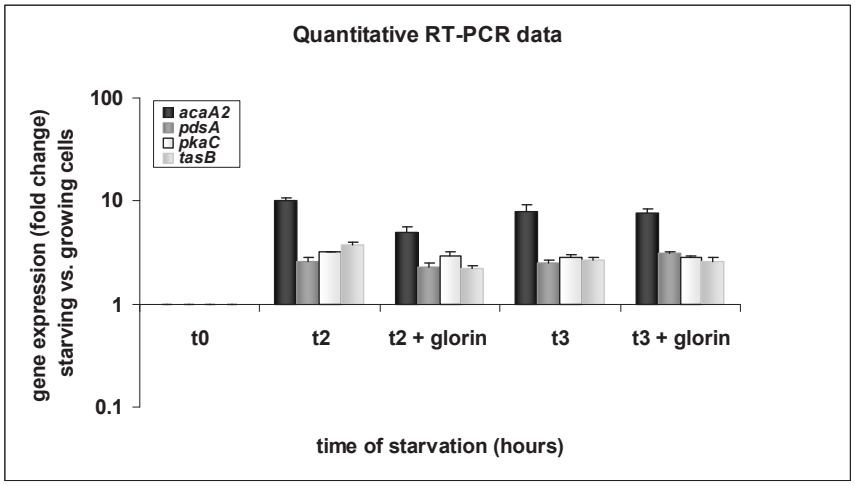


Figure 58: Expression kinetics of selected components of cAMP signalling in *P. pallidum*. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 amoebae (**t0**; fold change set to 1). Values > 1 represented higher expression of the gene in starving cells than in growing cells. As a control, expression of house keeping gene *gpdA* was examined. Cells were starved in shaking cultures for 2 or 3 hours in the absence of exogenous glorin treatment (**t2, t3**) or for 1 hour followed by 1 hour or 2 hours of glorin treatment (**t2+glorin, t3+glorin**). Relative expression of representative genes involved in cAMP signalling was determined by real-time RT-PCR. Mean values of triplicate measurements of the same cDNA ± SD were plotted.

3.9.2 *P. pallidum tasA*$^-$/*tasB*$^-$ null mutant exhibits normal aggregation

P. pallidum PN500 genome encodes two serpentine receptor genes named *tasA* and *tasB* that are highly homologous to *D. discoideum* cyclic AMP receptor genes. *tasA* is expressed strictly only after aggregates have formed, whereas *tasB* is expressed moderately during pre-aggregation and aggregation stages, while peak level expression is exhibited when aggregation has completed (Kawabe *et al.* 2009; Figure 59). In *P.*

pallidum PN500, *tasB* plays key role in normal fruiting body morphogenesis working together with *tasA*.

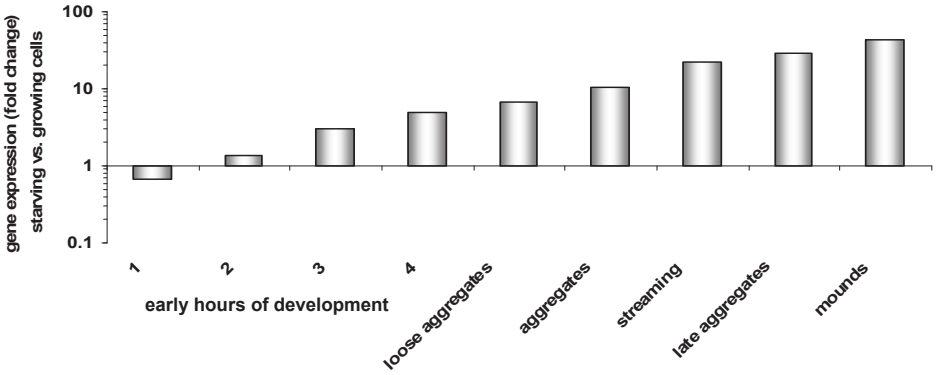


Figure 59: Temporal expression pattern of *tasB*. *P. pallidum* PN500 cells grown in association with bacteria were harvested in the late vegetative stage, washed, and resuspended in phosphate buffer. Cells were then starved on phosphate agar plates at a density of $8X10^5$cells/cm^2. Cell samples were collected at indicated developmental stages for total RNA extraction. Numbers 1, 2, 3, & 4 represent first four hours of development. Relative expression of *TasB* was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells where fold change was set to 1 and values > 1 represented higher expression of the gene in starving cells than in growing cells. Mean values of duplicate measurements of the same cDNA ± SD were plotted.

The *tasA*⁻*tasB*⁻ mutant exhibits astringent developmental aberrations; aggregation is normal, but after that instead of normal fruiting body formation, only small club-shaped structures are formed that are composed of thick lumpy stalks with spore heads containing cyst like cells rather than elliptical spores characteristic of normal fruiting bodies (Kawabe *et al.* 2009).

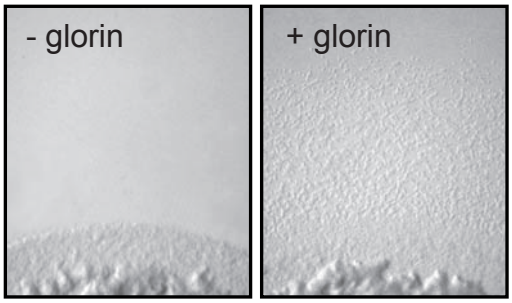


Figure 60: *P. pallidum* PN500 *tasA*⁻/*tasB*⁻ mutant exhibits normal chemotaxis towards glorin. Cells were starved in slow shaking buffer suspensions for 3 hours before 10 µl drops containing $2x10^5$ cells were plated on agar without (left picture) or with 1 µM final concentration of glorin (right picture). Pictures were taken 3 hours after plating.

Using chemotaxis assay adapted in this study (as described in Section 3.1), it was shown that *tasA*⁻*tasB*⁻ mutant displayed normal chemotaxis towards glorin (Figure 60) that is assumed as aggregative chemoattractant of *P. pallidum.* These results suggest that a functional cAMP signalling system is not invloved in mediating aggregation of *P. pallidum* amoebae.

Taken together, these limited data lead to speculate that in *P. pallidum* PN500 acrasin mediating aggregation and cAMP coordinating post-aggregation stages of development may function independent of each other. To get further insight into possible cross-talk between aggregation-specific glorin communication system and post-aggregative cAMP signalling, a future line of work would be to study glorin-regulated gene expression in *P. pallidum* PN500 mutants lacking individual components of cAMP signalling system.

3.10 Glorin elicits rapid changes in gene expression

Experiments conducted earlier in this study (Section 3.8.3) showed that when *P. pallidum* PN500 amoebae pre-starved for 1 hour were stimulated with glorin, expression of model genes PPL_09347 and PPL_05833 was either increased to high-levels (Figure 37 & 39) or decreased by high degree (expression kinetics of PPL_07908 shown in Figure 41) within 30 minutes of exposure to glorin. This phenomenon indicated that glorin induces rapid changes in gene expression in starving cells. It was therefore intriguing to characterize more deeply how fast the molecular response to glorin stimulation could be. In this experiment, collection of cell samples (for total RNA extraction) was narrowed down to 5 or 10 minutes of glorin treatment to evaluate rapidity of response.

P. pallidum PN500 amoebae grown in association with bacteria were harvested at late vegetative stage, washed free of bacteria and resuspended in phosphate buffer at concentration of $2x10^7$cells/ml. A pellet of $2x10^7$cells was stored at -80 ^oC that served as 'growing cells control' for successive gene expression analysis. Suspension of amoebae was pre-starved for 1 hour to initiate development and then equally divided into 2 flasks. Culture in one flask received 1 µM glorin pulses at 10-minute intervals for an additional one hour, whereas culture in second flask received no exogenous treatment. Cell samples were collected after 5-, 10-, 30- and 60-minutes of glorin treatment to assess how rapidly mRNA of model genes (PPL_09347 & PPL_05833; genes shown to be highly induced by glorin) accumulated in response to stimulation with exogenous glorin.

Samples were collected from untreated culture at the same time points. Total RNA was extracted to prepare cDNA for gene expression analysis using real-time RT-PCR. Relative gene expression of model genes PPL_09347 & PPL_05833 was determined in glorin-treated and untreated cells (Figure 61). Fold changes were calculated compared to expression in growing cells. As a control, expression of house keeping gene *gpdA* was examined.

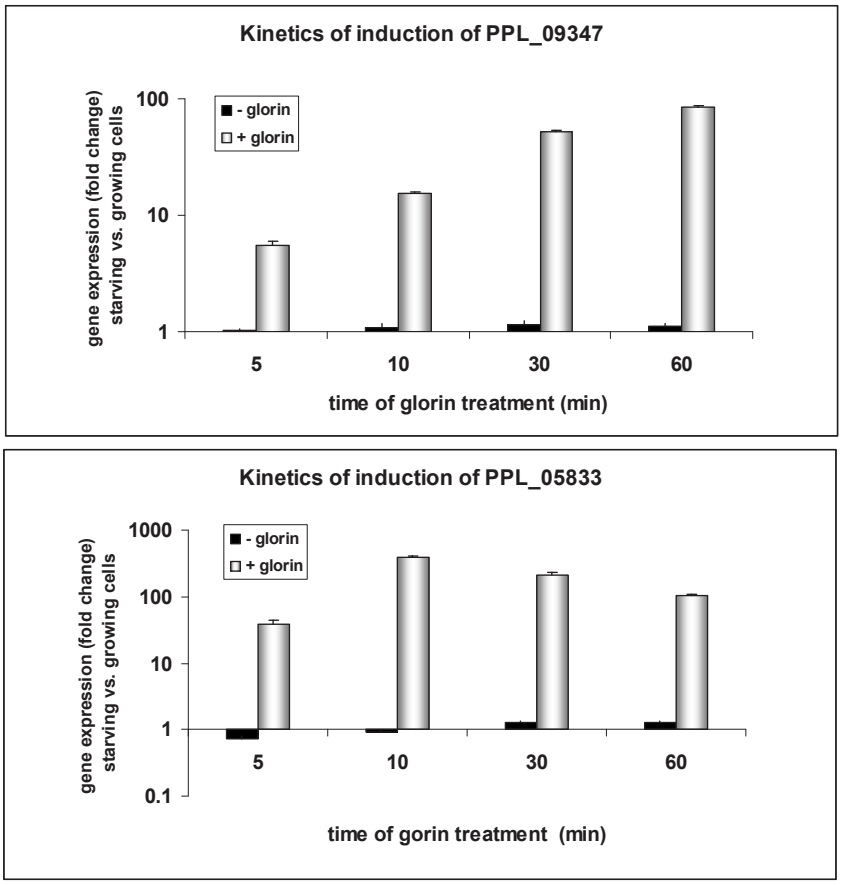


Figure 61: Time-course of PPL_09347 and PPL_05833 mRNA accumulation in untreated and glorin-treated cells. *P. pallidum* PN500 amoebae were pre-starved for 1 hour followed by treatment with 1 µM glorin at 10-min intervals for an additional 1 hour. Cell samples were collected after 5, 10, 30 & 60 minutes of glorin treatment. Samples harvested after 5 & 10 minutes of glorin treatment received only one pulse of glorin, whereas those collected at 30 & 60 minutes received 3 & 6 pulses of glorin, respectively. Cell samples were harvested from untreated cells at the same time points. Relative expression of PPL_09347 and PPL_05833 was analyzed using real-time RT-PCR. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells where fold change was set to 1 and values > 1 represent higher expression of the gene in starving cells than in growing cells. Values < 1 show that expression of gene is lower in starving cells than in growing cells. Mean values of triplicate measurements of the same cDNA ± SD were plotted.

It was noticed that the molecular response to glorin stimulation was very fast; within 5 minutes after addition of glorin, mRNAs of both model genes started to accumulate,

however, maximal levels of mRNA accumulation were found at different time points for PPL_09347 and PPL_05833 (Figure 61). Within 5 minute of glorin treatment, PPL_05833 and PPL_09347 were induced to 38- and 5.44-fold, respectively, compared to growing cells. PPL_09347 exhibited maximal mRNA level after 1 hour of glorin treatment, whereas mRNA of PPL_05833 accumulated to highest levels after 10 minutes of glorin treatment (Figure 61).

As shown in Figure 61, transcripts of PPL_05833 exhibited a gradual decline at 30- and 60-minutes of glorin treatment indicating that mRNA of PPL_05833 is relatively unstable. The non-conformity of the kinetics of PPL_09347 and PPL_05833 gene responses suggests the possibility that variations may exist in the mechanisms of regulation of different glorin-induced genes.

3.10.1 Pre-starvation period is not necessary to observe glorin-induced gene expression

In all experiments aimed to investigate glorin-regulated gene expression, *P. pallidum* PN500 cells were pre-starved for 1 hour prior to exposure to glorin. However, rapidity of response to glorin treatment (Figure 61) suggested that *de novo* protein synthesis may not be required to observe glorin mediated changes in gene expression in *P. pallidum* PN500 amoebae. This hypothesis prompted us to investigate responses to exogenous glorin in cells that were freshly washed free of bacteria, without any pre-starvation period, to understand whether or not starvation is necessary to observe glorin effects.

P. pallidum PN500 cells were harvested from bacterial growth plates at late vegetative stage, washed free of bacteria and resuspeneded in phosphate buffer at concentration of $2x10^7$cells/ml. This suspension was equally divided into 3 flasks. Culture in one flask was immediately pulsed with 1 µM glorin at 10-minute intervals for 1 hour without any pre-starvation period. Cells in flask 2 were pre-starved for 1 hour prior to receiving glorin pulses every 10-minute for an additional hour. Culture in flask 3 did not receive any external treatment and served as ‘untreated control’ for the time period. Cell samples (each containing $2x10^7$cells) were harvested from both un-prestarved and prestarved cultures after 10-, 30-, and 60-minute of glorin treatment for total RNA preparation. Samples were harvested from ‘untreated’ control culture at the same time points. Quantitative real-time PCR was employed to compare induction kinetics of PPL_09347 and PPL_05833 in un-prestarved and pre-starved cells (Figure 62).

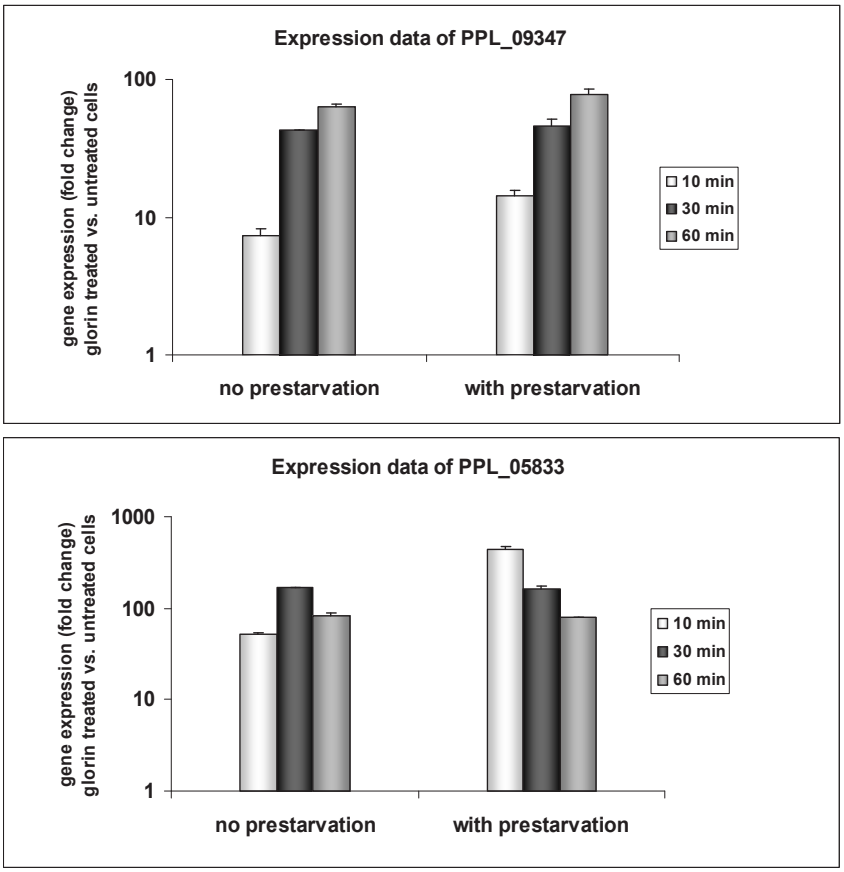


Figure 62: Comparison of induction kinetics of PPL_09347 and PPL_05833 in un-prestarved and prestarved cultures of *P. pallidum* PN500 cells following stimulation with exogenous glorin. Growing *P. pallidum* PN500 were harvested, washed to remove bacteria and resuspended in phosphate buffer at a density of 2X 10^7 cells/ml. Amoebae were then developed in shaking cultures in the presence or absence of glorin. Cells in 'no prestarvation culture' were immediately treated with 1 µM glorin at 10-minute intervals for 1 hour without any pre-starvation period. Culture labelled as 'with pre-starvation' was first starved for one hour before receiving glorin (1 µM) treatment every 10 minute for an additional hour. A control culture was maintained in the absence of glorin treatment. Cell samples were collected after 10-, 30- and 60-minutes of glorin treatment for total RNA extraction to analyse gene expression. Samples were collected from untreated cells at the same time points. As a control, expression of house keeping gene *gpdA* was examined. Relative expression of model genes PPL_09347 and PPL_05833 was determined by real-time RT-PCR. Fold changes are shown. All data were compared to untreated *P. pallidum* PN500 cells where fold change was set to 1 and values > 1 represented higher expression of the gene in glorin treated cells than in untreatedcells. Mean values of triplicate measurements of the same cDNA ± SD were plotted.

As shown in Figure 62, induction of PPL_09347 in response to exogenous glorin was comparable in both un-prestarved and 1 hr pre-starved cultures. PPL_05833 was also induced equally well in both cultures; however, an observed difference was comparatively higher induction of PPL_05833 after 10 minutes of glorin treatment in cells

that were pre-starved (Figure 62), indicating that starvation may increase sensitivity of cells towards initial exposure to glorin. Nevertheless, responses observed for PPL_05833 after 30- & 60-minutes of glorin treatment were consistent in both un-prestarved and pre-starved cells (Figure 62). Collectively, these data show that starvation is not required to observe glorin-regulated gene expression and supported the idea that prior protein sysnthesis may not required to see glorin responses. These results suggest that all components of an intracellular glorin signalling cascade are letently present in growing *P. pallidum* PN500 amoebae.

3.10.2 Glorin-regulated gene expression is not dependent on *de novo* protein synthesis

To verify the possibility that protein expression is not required for detection of gene regulatory effects of glorin, vegetative-stage *P. pallidum* PN500 cells were developed in shaking cultures in the presence or absence of cycloheximide that inhibits the elongation step of protein synthesis, followed by treatment with glorin. This approach was designed to assess the sensitivity of *'glorin-induced differential changes in gene expression'* to the cycloheximide that is known to inhibit the synthesis of proteins in related species *D. discoideum* (Clotworthy & Traynor 2006). In order to examine the sensitivity of *P. pallidum* PN500 cells to cycloheximide, a suspension culture of these cells ($2x10^7$cells/ml) was treated with 2 mM cycloheximide for 2 hours. A second culture was run under similar conditions in the absence of any external treatment (no cycloheximide added) for the time period and served as 'control'. Cycloheximide treated and untreated cells were then plated for development on non-nutrient agar plates at a density of $8x10^5$cells/cm^2 at 21^{o}C. It was observed that cycloheximide-treated cells exhibited 12 hours delay in development compared to untreated cells (data not shown), indicating that *P. pallidum* PN500 cells are sensitive to cycloheximide in the same manner as are the *D. discoideum* amoebae.

Growing *P. pallidum* PN500 cells were harvested, washed and suspended in phoshphate buffer at cell density of $2x10^7$cells/ml. A pellet of $2x10^7$cells was stored at -80^{o}C and served as 'growing cells control' for consecutive gene expression analysis. Cell suspension was equally splitted to 4 flasks. Culture in *flask 1* did not receive any external treatment (- cycloheximide - glorin) and acted as control for the time period. Cells in *flask 2* were incubated with 2 mM cycloheximide immediately at the onset of starvation for 30 minutes and then maintained in the absence of glorin treatment for an

additional 60 minutes (+ cycloheximide - glorin); this culture was prepared to detect effects of only cycloheximide treatment. Culture 3 was pre-starved for 30-minutes in the absence of cycloheximide treatment followed by exposure to 1 μM glorin at 10-minute intervals for 60 minutes (- cycloheximide + glorin); this culture was prepared to observe the effects of only glorin treatment. *Flask 4* culture was incubated with 2 mM cycloheximide instantly at the onset of starvation for 30 minutes prior to the addition of 1 μM glorin every 10 minute for an additional 60 minutes (+ cycloheximide + glorin); this culture was prepared to examine gene regulatory effects of glorin in cells treated with cycloheximide. All cultures were developed under slow shaking conditions (i.e. 100 rpm). Cell samples were collected from glorin treated cultures (flask 3 & 4) after 10-, 30-, and 60-minutes of exposure to glorin. Samples were harvested from culture 1 & 2 (not treated with glorin) at the same time points. Total RNA was extracted and quantitative RT-PCR was employed to study induction kinetics of two model genes PPL_09347 and PPL_05833 in untreated cells (i.e. control) and cells treated either with glorin or cycloheximide or both glorin and cycloheximide. All data were compared to gene expression in growing cells (Figure 63).

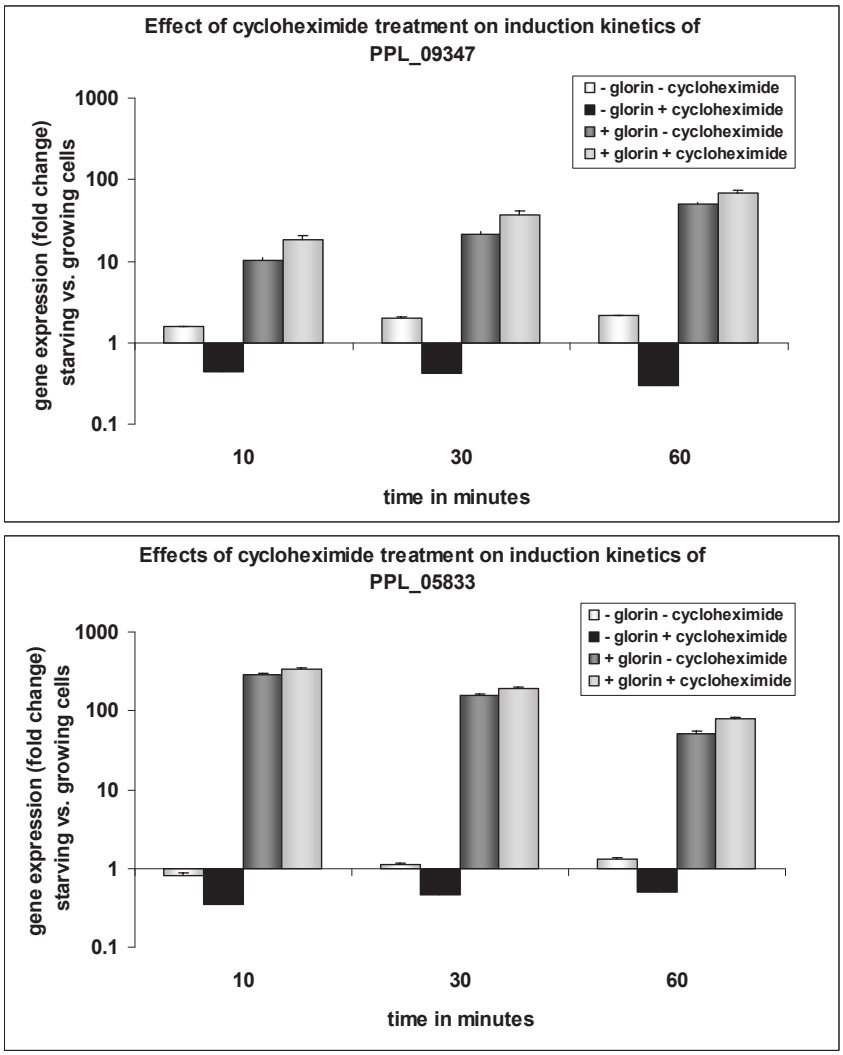


Figure 63: Glorin-induced gene expression is insensitive to cycloheximide. *P. pallidum* PN500 cells were harvested from bacterial growth plates, washed and resuspended in phosphate buffer. The cell suspension was splitted into 4 flasks, each of which received one of the following treatments: (i) no additions (ii) 2 mM cycloheximide for 30 minutes immediately at the onset of starvation (iii) pre-starved for 30 minutes followed by addition of 1 µM pulses of glorin delivered at 10-min intervals for an additional 60 minutes (iv) pre-treatment with 2 mM cycloheximide for 30 minutes prior to addition of 1 µM glorin every 10- minutes for an additional 60 minutes. From glorin treated cultures, cells were sampled after 10, 30 & 60 minutes of exposure to glorin. From untreated or only cycloheximide treated cultures, cell samples were collected at the same time points. Total RNA was extracted ans relative expression of model genes PPL_09347 and PPL_05833 was determined by real-time RT-PCR. As a control, expression of house keeping gene *gpdA* was examined. Fold changes are shown. All data were compared to growing *P. pallidum* PN500 cells with fold change set to 1 where values > 1 represented higher expression of the gene in glorin treated cells than in growing cells. Values < 1 represented lower expression of the gene in glorin treated cells than in growing cells. Mean values of triplicate measurements of the same cDNA ± SD were plotted.

It was found that in control cells (shaken in the absence of added glorin and cycloheximide), PPL_09347 was expressed to low levels, whereas PPL_05833 exhibited only basal level expression (Figure 63). A short incubation of cells in cycloheximide (in the absence of glorin treatment) led to down-regulation of both genes, indicating that even slight induction of model genes in response to starvation was inhibited by cycloheximide (demonstrating that starvation-induced expression of PPL_09347 and PPL_05833 may require *de novo* protein synthesis). However, cells treated only with glorin showed precocious induction of both PPL_09347 and PPL_05833 (Figure 63). Interestingly, it was observed that amoebae pre-treated with cycloheximide retained their ability to respond to externally added glorin. Cultures shaken in the presence of cycloheximide showed no detectable changes in glorin-induced gene expression, instead expression of PPL_09347 and PPL_05833 was somewhat enhanced in cells treated with both cycloheximide and glorin. This phenomenon may reflect that cycloheximide stabilizes mRNA signal that would otherwise be degraded at this time in development. These data indicate that glorin-induced changes in gene expression do not require *de novo* protein synthesis.

3.11 Glorin induces aggregation sensitivity in starving *P. pallidum* cells

Aggregation is the first step in the transition from growth to development of *Dictyostelids*. *Polysphondylium* amoebae aggregate towards collecting centres that would develop spontaneously (Shaffer 1957b). Upon starvation, a few of the randomly moving amoebae come to rest and start acting as centres on which the others converge. Such centres are known to secrete acrasin (Bonner 1949). Sensitivity to acrasin begins to develop slowly throughout the population as centres appear.

In the present work, it is shown that exposure of *P. pallidum* PN500 cells to glorin prepared starving cells for aggregation precociously, compared with control cells that were not treated with glorin. To examine stimulatory effects of glorin on aggregation capacity of amoebae, vegetatively growing *P. pallidum* PN500 cells were harvested, resuspended in phosphate buffer at a cell density of $2x10^7$cells/ml, and pre-starved for 1 hour. Suspension of amoebae was then divided into three parts. One culture was pulsed with 100 nM glorin (final concentration) at 10 minute intervals for 2 hours. A second culture was treated with 1 µM glorin (final concentration) added every 10-minutes for 2 hours. A control culture was run under similar conditions where cells did not receive any

exogenous treatment. Cells were then harvested by centrifugation and plated for development as monolayers on non-nutrient agar plates at a density of $8x10^5$cells/cm^2.

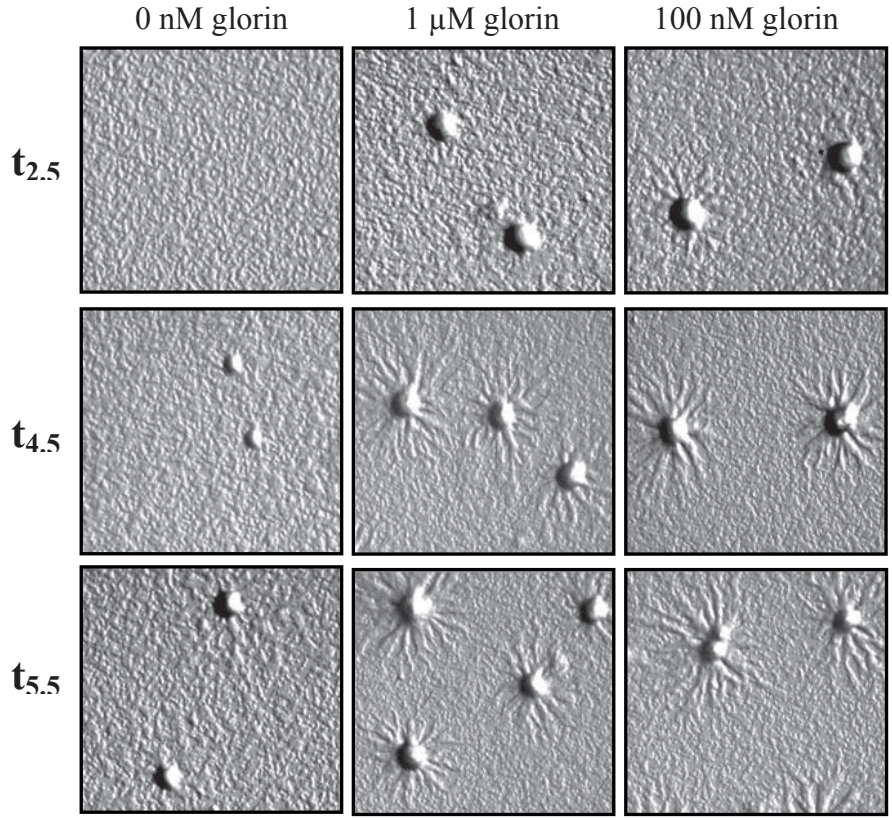


Figure 64: Developmental phenotypes of glorin treated and untreated cells. *P. pallidum* PN500 cells were first pre-starved for 1 hour in shaken suspensions and then stimulated with 0nM, 1 µM and 100 nM glorin at 10-min intervals for 2 hours in three separate cultures. Amoebae were then harvested from shaking cultures and plated on non-nutrient agar plates and photographed when first signs of aggregation were seen in glorin-treated cells (middle and right pictures). Photographs were captured for untreated cells at the same time points, shown in the left picture. Cells were equally sensitive to both concentrations of glorin i.e. 1 µM and 100 nM applied as pulses. At 2.5 hours ($t_{2.5}$) after plating, the centres have grown considerably in glorin stimulated cells, though still almost devoid of streams indicating that glorin stimulates rate of aggregation in starving cells. At the same time there was no sign of centre formation in untreated cells ($t_{2.5;}$ left picture).

It was observed that glorin treatment sensitized cells and they gained ability to initiate centres precociously as compared to untreated cells. Appearance of some primary centres was noticed in glorin treated cells at approximately 2.5 hours ($t_{2.5}$) after plating, whereas at the same time point untreated cells showed no signs of centre formation (Figure 64). Amoebae stimulated with glorin exhibited streams of cells converging on the aggregation centres at 4.5 hour (Figure 64; $t_{4.5}$). In comparison, centres just started to appear in untreated cells at that time ($t_{4.5}$ hour), implying that initial formation of aggregation centers was delayed by 2 hours (Figure 64). By 5.5 hours (Figure 64; $t_{5.5}$),

some more aggregation centers were visible in untreated cells. The glorin-treated cells congregated more efficiently into aggregates, indicating that sensitizing cells with glorin switched aggregation competence to earlier time points. No pronounced difference in responsiveness of cells was observed with two different concentrations of glorin employed for pulsing (i.e. 100 nM and 1 µM final concentration of glorin). At 5.5 hours ($t_{5.5}$) centres began to grow in untreated cells. These data suggest that glorin accelerates cell aggregation; most probably by precociously inducing expression of several aggregation specific genes.

4 Discussion

One of the major transitons in evolution is the emergence of multicellular organisms from single cells (Mian & Rose 2011). Multicellularity apparently evolved through clonal development (from a unicellular spore or zygote) or aggregation of scattered cells (Siu *et al.* 2011; Grosberg & Strathmann 2007; Bonner 1998). Aggregative development of multicellularity takes place in a few groups of microorganisms, including myxobacteria, myxomycetes, and *Dictyostelids* (Siu *et al.* 2011; Grosberg & Strathmann 2007; Bonner 2000). A fundamental requirement for aggregative multicellular organization is cell-cell communication that coordinates cellular behaviour. The unique life cycle of social amoebae shifting between unicellular and multicellular stages offers an ideal system to study complex process of intercellular communication (Chisholm & Firtel 2004; Annesley & Fisher 2009). These organisms live independently for most of their life cycles, but aggregate to become multicellular in response to starvation, constructing a fruiting body within 24 hours. So far, intercellular communication has been extensively studied in the model organism *D. discoideum* that uses cAMP language for cell-cell interaction to organize the process of aggregation.

Glorin is a peptide chemoattractant employed by *P. violaceum* amoebae for cell-cell communication to coordinate the process of aggregation. It was proposed that glorin mediated communication might be wide-spread among the *Dictyostelids* (Schaap *et al.* 2006). In the first part of this study, responses to glorin were studied in all 4 phylogenetic groups of *Dictyostelids* defined by Schaap *et al.* (2006) to identify evolutionary roots of peptide communication in *Dictyostelids*. In the second part of this research work, specific cellular responses to glorin such as glorin-mediated changes in gene expression patterns and developmental effects of glorin were studied in the genetically tractable species *P. pallidum* PN500.

4.1 Glorin is an ancient extracellular messenger molecule used for intercellular communication in *Dictyostelids*

P. violaceum amoebae start to secrete glorin when the aggregation process begins to stimulate surrounding cells of the same species that can sense glorin signal by specific cell surface receptors and move chemotactically toward glorin gradients generated by the glorinase activity of these cells (De Wit *et al.* 1988; Wurster *et al.* 1976). Thus, *P. violaceum* cells possess the requisite biochemical machinery to use glorin as acrasin. To

explore how outspread glorin communication is among *Dictyostelids*, a modified small population chemotaxis assay was used (Section 3.1) and responses to glorin of a collection of species that span the dictyostelid phylogeny were examined (Table 1). All tested *Polysphondylid* species from early diverged group 2 reacted well to glorin in this study (Figure 16). Two dictyostelid taxa *D. gloeosporum* and *D. oculare* placed in group 2 were also equally responsive to glorin (Figure 16). This study further showed for the first time that group 1 *Dictyostelids* are chemotactically reactive to glorin in a very similar manner as the *Polysphondylids* (Figure 17). However, none of the tested *Acytostelium* species responded to glorin.

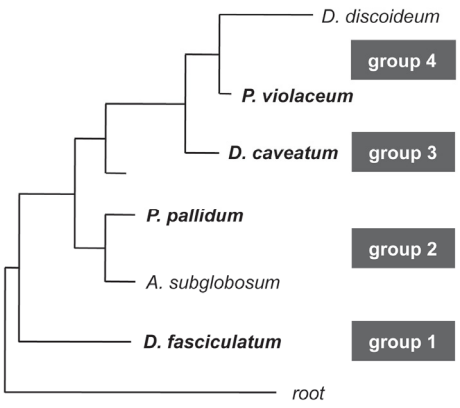


Figure 65: A simplified phylogenetic tree summarizing glorin chemotaxis by social amoebae. This tree is rooted according to (Schaap *et al.* 2006) and is not drawn to scale. The four major groups of social amoebae are illustrated by a single representative species investigated in this study (except for *Dictyostelium caveatum*, which was reported to respond to glorin by Waddell (1982). Species that show chemotaxis to glorin are indicated in bold.

Thus, this study uncovered that glorin-based cell-cell signalling is a common feature of species from the early diverging group 1 and 2 of *Dictyostelids* and therefore, the oldest form of intercellular communication used at the transition from growth to multicellular development of social amoebae. Previously, *D. caveatum*, belonging to group 3 in the molecular phylogeny of *Dictyostelids* (Schaap *et al.* 2006), has been noted to respond chemotactically to glorin (Waddell 1982b). Chemotactic aggregation of individual amoebae of *P. violaceum*, which is more related to the most derived group 4 species (Romeralo *et al.* 2011b; Schaap *et al.* 2006) than to other group 2 *Polysphondylids*, was shown to be mediated by glorin (Shimomura *et al.* 1982). Nevertheless, none of the tested group 4 species was reactive to glorin. Thus, a striking finding of this research work is that in the course of evolution, social amoebae either lost or abandoned the use

of glorin as intercellular communication molecule in group 2 Acytostelia, some group 3 species and all group 4 *Dictyostelids* (Figure 65).

In some of these taxa, glorin-mediated cell-cell signalling was replaced by communication systems based on cellular metabolites such as cAMP and folate derivatives. The molecular phylogeny introduced by Schaap *et al.* (Schaap *et al.* 2006) anticipated that group 1 *Dictyostelids* preceded group 2 taxa, though an alternative root organizing group ½ and ¾ in a paraphyletic clade cannot be ruled out. Complete nuclear genome sequences of *D. discoideum*, *P. pallidum* PN500, and *D. fasciculatum* (Heidel *et al.* 2011) and the mitochondrial genomes of these species (Heidel & Glöckner 2008) encourage the latter possibility. Whatever the origin of *Dictyostelids* is, data presented in this dissertation strengthen the assumption that the unknown last common ancestor of all social amoebae used glorin to coordinate the transition from uni-to multicellularity.

Critical evaluation of the positive chemotactic response of group 1 and 2 species (except Acytostelids) to glorin suggests that the cells of these species are capable of sensing chemoattractant and detect its gradient. It is intriguing that sensitivity to glorin is highest just before aggregation of responding species. These facts support that glorin may be the acrasin of responding species. However, at this stage of research, chemotaxis of amoebae towards glorin does not identify this dipeptide as the chemical messenger responsible to organize aggregation. Therefore, future studies should focus on the isolation of glorin from aggregating amoebae of group 1 and 2 species to confirm the presumption that glorin is indeed the acrasin of these species. The identification of some components of the glorin signalling pathway, most importantly glorinase(s), cell surface glorin receptor and enzymes involved in glorin biosynthesis in the genetically amenable species *P. pallidum* PN500 may lead to further elucidation of the process of chemotaxis and cell aggregation in this species, whereas comparison with other genetically tractable dictyostelid species will provide interesting information about possible functions of glorin-signalling system genes in species that no longer use glorin as acrasin.

4.2 Stage specific responses of *P. pallidum* PN500 cells to exogenous glorin

In some experiments that were designed to study gene regulatory effects of glorin, considerable variations were noticed in the cellular responses to exogenous glorin when different batches of *P. pallidum* PN500 cells were compared with each other (compare expression levels of PPL_05833 after 2 hours of glorin treatment in Figure 27 & Figure

39). This phenomenon can be explained by the fact that, despite great care, the populations of cells used in these experiments were never completely synchronized. Insufficient synchronization results in substantial variability in the number of glorin receptors per cell (De Wit *et al.* 1988), therefore, responses to externally added glorin varied significantly between experiments. It was also observed that *P. pallidum* PN500 cells were sensitive to the effects of exogenous glorin only within a certain period during early hours of development, whereas responses decline once cells have crossed that sensitivity stage (data not shown); most probably because cells establish endogenous glorin signalling with progression in development, therefore responses to exogenous glorin diminish.

4.3 Glorin mediates rapid changes in gene expression during early development of *P. pallidum*

D. discoideum cells exhibit profound changes in transcription as they undergo growth- to aggregation transition (Iranfar *et al.* 2003; Van Driessche *et al.* 2002). Extracellular cAMP pulses not only act as a chemoattractant to organize aggregation but also to induce optimal expression of genes whose activity is required for the aggregation process, such as genes encoding aggregation-stage cAMP receptors, adenylate cyclase A, extracellular cAMP phosphodiesterase PdsA (Schaap 2011a; Alvarez-Curto 2001; Pitt *et al.* 1993; Darmon *et al.* 1975; Gerisch *et al.* 1975) and the cell adhesion molecule gp80 (Desbarats *et al.* 1992; ; Kumagai *et al.* 1991; Ma and Siu 1990; Mann & Firtel 1989; Kumagai *et al.* 1989; Kamboj *et al.* 1989, 1988; Mann & Firtel 1987; Noegel *et al.* 1986; Wong & Siu 1986), whereas many preaggregation stage genes are repressed by cAMP pulses (Mann & Firtel 1989).

In this study, using high-throughput RNA-seq technology it was shown that the treatment of *P. pallidum* PN500 cells with glorin led to dramatic changes in gene expression patterns during first few hours of starvation (Section 3.6.2; Figure 24). It was observed that glorin not only *induced* expression of a number of genes but also *enhanced* expression of many starvation-induced genes. However, several other genes were *repressed* in response to glorin treatment including some of the genes that were, otherwise, *induced* by starvation. Products of genes whose expression was *induced* or *enhanced* by glorin, might play important roles in the aggregation process. Search of the putative molecular functions for glorin-induced genes, based on homologies to annotated *D. discoideum* genes, indicated that many genes upregulated by glorin may play roles in

signal transduction pathways, reorganization of actin cytoskeleton during chemotaxis, reprogramming of the metabolic machinery, gene transcription and protein translation, cell adhesion, degradation of nuclear DNA, secretory pathways, transportation of metabolic products, lipids and toxins across extra- and intracellular membranes, cell recognition and transcription repression (Table 4; section 3.7.1).

4.4 Rapid turn-over of transcripts of glorin-induced genes

RNA-seq analysis demonstrated that glorin-mediated induction/enhancement of expression of several genes was transient (Section 3.6.2). When shaken suspensions of *P. pallidum* PN500 cells were stimulated with glorin for 1 hour, a more than 3-fold differential expression of 115 genes, 70 of which were upregulated by 3- to 57-fold was observed. When cells were treated with glorin for an additional hour, 120 genes were differentially expressed, but only 20 of them were upregulated more than 3-fold (Section 3.6.2; Figure 24). It was noticed that 133-, 16- & 12-fold increase in the mRNA levels of PPL_05833 (Figure 27), PPL_12248 and PPL_12249 (Figure 29), respectively, seen after 1 hour of glorin treatment was not maintained even if pulses of glorin were given frequently at short intervals (Figure 39; Figure 42; Figure 43). Consequently, transcript levels of these genes declined significantly after 2 hours of exposure to exogenous glorin. This behaviour indicates a rapid mRNA turn-over of glorin-induced gene transcripts. Studies suggest that in mammalian cells a large percentage of activation-induced transcripts have short mRNA half-lives (Raghavan & Bohjanen 2004; Frevel *et al.* 2003; Tebo *et al.* 2003; Raghavan *et al.* 2002; Lam *et al.* 2001); demonstrating that cells have a coordinated strategy to rapidly modulate the mRNA levels of these genes (Lam *et al.* 2001). A similar situation was described for the regulation of prolactin gene transcription by thyrotropin-releasing hormone (TRH) in the GH4 rat pituitary cell line, where mRNA levels started to decline within 1 hour after initial rapid induction of prolactin gene expression by TRH and further addition of thyrotropin-releasing hormone resulted in no additional response during this time (Saski *et al.* 1984; Murdoch *et al.* 1983). The 'burst-attenuation' kinetics of expression of genes in response to glorin may be due to a common molecular mechanism at the gene level. The inducibility of many of these genes may be due to transcriptional activation of their promoters and the rapid degradation of these transcripts permits their levels to rapidly drop when transcriptional activation is terminated. Assuming that glorin is the acrasin of *P. pallidum*, and then under natural conditions, expression of these genes might be stably induced/enhanced by glorin in the aggregation process, when products of these genes are actually required. This

assumption was verified by examining developmental expression kinetics of a number of glorin-regulated genes in cells starved on agar surface. These experiments showed that most of the genes 'transiently upregulated by glorin under shaking conditions' were stably expressed in aggregating cells during development on agar (Figure 26 & 46, Figure 48 & 26, Figure 27 & 50; Figure 51 & 26; Figure 53 & 27; Expression kinetics of PPL_06644 depicted in Figure 47 & Table 3); indicating that cell-cell contacts may be necessary for stable expression of these genes. Under artificial shaking culture conditions, externally applied glorin induced these genes or enhanced their expression precociously (before cells have gained aggregation competence); therefore at this time in development, transcripts of most of these genes were unstable and degraded promoting rapid turn-over of these genes. Recently, it was reported that mRNA decay plays an important role in regulating transient gene expression events in mammalian cells (Raghavan & Bohjanen 2004). Another explanation of rapid decrease in certain mRNA abundances may be that excess of these transcripts are accumulated in mRNA-protein complexes at this stage of development; though mRNA sequestration has not generally been recognized as a major factor in the dynamics of mRNA abundance (Aragon *et al.* 2006). Yet another justification of the rapid changes in mRNA levels of many genes may be that increases in translation rates lead to decreases in transcript abundance (Aragon *et al.* 2006). Hence, by regulating mRNA turnover, cells can selectively and efficiently maintain or diminish their gene expression programme appropriate for a specific stage of development.

4.5 Glorin-mediated gene repression

When *P. pallidum* PN500 cells in shaken suspensions were stimulated with exogenous glorin for 1 hour, 45 genes were down-regulated between 3- and 17-fold (Figure 24; Section 3.6.2). However, after 2 hours of exposure to glorin, 100 genes were down-regulated (Figure 24; Section 3.6.2). A reasonable justification of this difference may be the rapid kinetics of glorin effects. Similar phenomenon was observed in microarray-based studies of primary human T cells where almost 400 transcripts that were stable in resting T cells became destabilised following T cell receptor-mediated stimulation of cells and their steadystate levels were repressed (Raghavan *et al.* 2002). It was noticed that 80% of the genes down-regulated after two hours of glorin treatment were actually downregulated one hour earlier also, but they did not reach the 3-fold change threshold (Section 3.6.2). Stimulating *P. pallidum* cells with exogenous glorin starting at 1 hour of starvation was an unnatural experimental arrangement. Apparently, this approach

interfered with the normal developmental cycle and the associated gene expression changes. In wild-type cells, under natural conditions, glorin may be secreted by cells at the onset of the aggregation process to mediate relevant gene expression changes including repression of early developmental genes, induction of aggregation-stage genes and enhancement of expression of many other starvation induced genes. However, when *P. pallidum* PN500 cells in shaking cultures were treated with 1 µM glorin at 30-minute intervals during very early hours of starvation (Section 3.6), normal physiological concentrations of acrasin and natural timings of its effects do not exist when correlated to wild type cells. Most probably, treating *P. pallidum* amoebae with glorin conditioned them in a way that either the starvation responses of the signalling competent cells were enhanced precociously or these responses were shifted to earlier time points in the developmental cycle. Therefore, repression of many genes observed in response to glorin treatment during early hours of starvation may be required for proceeding to aggregation under normal physiological conditions. Apparently, stimulation of cells with glorin triggered signals that altered the cellular mRNA degradation machinery, leading to specific transcript destabilisation. Recently, it was suggested that mRNA decay may be an important mechanism for gene repression in an activation-dependent manner (Raghavan & Bohjanen 2004). A recent study by Frevel and colleagues (Frevel *et al.* 2003) suggested that p38 mitogen-activated protein kinase (MAPK) plays critical role in the regulation of the decay of a variety of transcripts in THP-1 monocytes. Annotation of GO terms to glorin-repressed genes was not very explanatory because many of these genes have not yet been assigned to any specific functions (Section 3.7.2). However, a number of genes repressed by glorin may encode products that are constituents of the metabolic machinery (Appendix Table A7 and A8). Future studies focusing on the identification and subcellular localization of the proteins encoded by glorin repressed genes will help to understand the functional roles of these genes.

4.6 Allosteric or covalent modification of existing proteins may mediate rapid effects of glorin

In this study, it is shown that mRNAs of model glorin-induced genes started to accumulate within 5 minute after the addition of glorin (Figure 61; Section 3.10). Similar rapid kinetics of induction has been reported for P-enolpyruvate carboxykinase gene transcription in H4IIE cells (differentiated rat liver cells) in response to stimulation with cAMP analogs (Sasaki *et al.* 1984); transcription of multiple genes after the addition of beta interferon to human fibroblasts or to HeLa cells (Larner *et al.* 1984); prolactin gene

transcription in pituitary cells on exposure to cAMP (Preston *et al.* 1990; Sasaki *et al.* 1984; Murdoch & Rosenfeld 1982b) and the yeast *Saccharomyces cerevisiae* responding to diverse environmental transitions (Aragon *et al.* 2006; Martinez *et al.* 2004; Newcomb *et al.* 2003).

Apparent rapid increase in steady-state mRNA levels of a number of genes in response to glorin treatment (Figure 24; Appendix Table A4, A5 & A6) may result from a combination of *de novo* transcription, stabilization of mRNA, or potential release of extraction-resistant mature mRNA present in cells in a rapidly releasable form, possibly sequestered in protein complexes. Formation of such mRNA-protein complexes has been reported in yeast, mammalian and plant cells (Aragon *et al.* 2006; Kedersha & Anderson 2002). Release of the sequestered mRNAs upon exposure of cells to glorin may be a mechanism that allows cells to respond to environmental conditions as quickly as possible, in preparation for the activation of transcription and translation.

Regulated mRNA turn over upon extracellular stimulation is a critical process in control of gene expression in eukaryotic cells. Recently, it has been shown that mRNA stability is modulated by signal transduction pathways involving phosphorylation events (Sinsimer *et al.* 2008; Knapinska *et al.* 2005; Shim & Karin 2002). In mammalian cells several signalling pathways are involved in regulating the stability of mRNAs including activation of protein kinase C, Phosphatidylinositol 3-kinase (PI3K), and the mitogen activated protein kinases, elevation of intracellular Ca^{2+}, c-Jun N-terminal protein kinase (JNK) and p38 (Shim & Karin 2002). In *D. discoideum* YakA protein kinase regulates stabilization of *pka*C mRNA by inhibiting the expression of PufA protein (Souza *et al.* 1999, 1998).

Seemingly, the rapidity of the effects of glorin, and the observation that de novo protein synthesis is not required may indicate that glorin is directly affecting transcription of 'glorin-regulated genes' and the signal generated by binding of glorin to cell surface receptors rapidly reaches the cell nucleus. A protein phosphorylation-dephosphorylation mechanism appears to be an obvious possibility that may require either the translocation of a protein kinase and/or protein phosphatase from the cytoplasm to the nucleus, or the translocation of a regulatory phosphoprotein to the nucleus, or the presence of a cAMP-dependent protein kinase/protein phosphatase system in the nucleus. It has been shown that in mammalian cells hormones or neurotransmitters acting though G-protein coupled receptors activate second messenger pathways that in turn regulate the phosphorylation of specific nuclear proteins, leading to change in gene expression (De Cesare D &

Sassone-Corsi P 2000; Montminy 1997; Lalli & Sassone-Corsi 1994; Sassone-Corsi 1994). Previously, it was proposed by Jungmann and colleagues (Jungmann *et al.* 1983) that isoproterenol induces a cAMP-dependent protein kinase to translocate into the nucleus of C6 glioma cells that results in the phosphorylation of histones and/or RNA polymerase II. If such a mechanism is involved then the substrate of phosphorylation is of principle importance. Rosenfeld and coworkers reported that stimulation of GH4 cells with cAMP or thyrotropin-releasing hormone leads to rapid phosphorylation of a chromatin-associated basic protein, and this modification occurs prior to the increase of prolactin gene transcription (Murdoch *et al.* 1983; Murdoch *et al.* 1982a; Murdoch & Rosenfeld 1982b). It has been shown that addition of 8-CPT-cAMP to H4IIE rat pituitary cells causes full activation of protein kinase in less than 1 minute (Murdoch & Rosenfeld 1982b), therefore, on kinetic grounds, protein kinase activation may be involved in mediating effects of extracellular glorin on gene expression. However, transcription induction mechanism may also involve proteins that possess domains containing intracellular cAMP inducible activities which are independent of direct phosphorylation by protein kinase A (PKA).

Results of this research work pointed that stimulation of *P. pallidum* cells with glorin not only rapidly induced a number of genes but also predominantly ‘enhanced’ expression of many genes that were normally induced by starvation to very low levels (Section 3.6.2.1.3; Figure 27) during very early hours of development. If *de novo* transcription is involved in increasing transcript levels of glorin-regulated genes, then glorin-mediated transcriptional activation appears to be a highly ordered process. It is plausible that an inactive RNA polymerase II holoenzyme complex is constitutively positioned and maintained on the promoters of many genes that are rapidly induced in response to glorin stimulation. Therefore, *P. pallidum* cells appear to be programmed to increase the abundance of transcripts of these genes in response to glorin under any condition. This research work led us to propose that stimulation of cells with exogenous glorin triggers a cascade of intracellular phosphorylation events, which may lead to phosphorylation and subsequent translocation of a regulatory protein from the cytoplasm to the nucleus where it binds to sequence-specific enhancer elements in the promoter/enhancer regions of glorin-regulated genes. The assembled ‘enhanceosome’ may then recruit the transcriptional coactivator, which binds in a complex with the RNA polymerase II holoenzyme, allowing rapid transcriptional induction in response to glorin. It is suggested that the nuclear regulatory protein may be constitutively expressed but its function is acutely sensitive to glorin-mediated intracellular signalling cascade. During growth or late

development, when glorin signalling does not occur, the trans-acting protein may be quiescent.

4.7 Dose-response effects of glorin on gene induction

To determine whether starving amoebae are capable of detecting differences in the strength of the glorin stimulation, mRNA levels of model glorin-induced genes were compared in cells that were subjected to increasing doses of glorin. Increased doses of exogenous glorin resulted in relative increased levels of transcripts of PPL_09347 and PPL_05354 genes (Figure 30). Therefore, the ability to detect the strength of stimulation and to transmit quantitatively variable signals is a feature expected of the machinery that perceives glorin stimulus. Moreover, the regulatory mechanism governing the expression of PPL_09347 and PPL_05354 does not seem to be an on-off switch, but must act proportionally to a quantitative signal.

4.8 General kinetics of gene induction in response to repetitive stimulation of *P. pallidum* PN500 cells with glorin remains the same

Experiments aimed to determine the optimal glorin concentration and pulsing frequency required to observe maximal response by *P. pallidum* PN500 cells indicated that the general time-course of glorin activity was not dependent on the glorin concentration or pulsing frequency applied, only the level of induction was lower at reduced glorin concentrations and vice versa. When *P. pallidum* PN500 cells were treated with 10, 100, or 1000 nM glorin at 30 min intervals, it was found that 10 nM glorin was sufficient to induce expression of model genes PPL_09347 and PPL_05354 (Figure 30). Induction was significantly stronger at 100 nM, but most pronounced at 1000 nM glorin (Figure 30). In order to evaluate whether the frequency of glorin pulses influenced the induction of model genes, when 1 µM or 100 nM of glorin was applied for 4 hours at 10 and 30 minute intervals, it was found that repetitive pulsing resulted only in a stronger response, whereas general kinetics of induction remained the same (Figure 32 & 34). Also it was noticed that even a single pulse of glorin could induce model genes like PPL_09347 and PPL_05833 to almost the same level as observed after repeated pulsing (Figure 32 & 34). Generally, repetitive stimulation is supposed to result in a response that is stronger and more pronlonged and though amoebae are capable to respond to intermittent repititive stimulation, partial desensitization or habituation may occur.

4.9 Stimulation of *P. pallidum* PN500 cells with glorin induces precocious aggregation

It was noticed that *P. pallidum* PN500 cells treated with glorin displayed accelerated aggregation (Figure 64). Apparently, exogenous glorin signal triggered a cascade of intracellular biochemical events that led to the precocious induction of a number of genes whose products may participate in the aggregation process while repressing expression of several other early developmental genes. Considerable number of glorin-induced genes may facilitate cell-cell interactions causing formation of many small aggregates earlier than under natural conditions. Correspondingly, previous studies reported precocious aggregation of *D. discoideum* cells that were treated with 30 nM cAMP for 5 hours to mimic the normal oscillatory pulses of cAMP that occur during aggregation (Mu *et al.* 1998; Ma *et al.* 1997; Insall *et al.* 1996; Louis *et al.* 1993; Saxe *et al.* 1991a,b; Mann *et al.* 1988; Kimmel 1987; Mann & Firtel 1987; Janssens & van Haastert 1987; Devreotes 1982; Juliani *et al.* 1981; Darmon *et al.* 1975; Gerisch *et al.* 1975b; Gerisch 1968).

4.10 Pre-starvation is not needed to observe glorin-induced changes in gene expression

In vegetatively growing *D. discoideum* amoebae, the cAMP signalling system is expressed at very low levels. It has been known for some time that *D. discoideum* cells express many developmental genes while suspended in buffer and treated with pulses of cAMP at 6-min intervals for 2-6 hours (Iranfar *et al.* 2003; Louis *et al.* 1993; Pitt *et al.* 1993; Mann & Firtel 1989; Kumagai *et al.* 1989; Mann & Firtel 1987), reflecting that developmentally regulated synthesis of a protein(s) is required for induction of these genes. Contrarily, in *P. pallidum* PN500 amoebae all components of glorin signalling appear to exist in cells prior to the onset of starvation because even freshly washed cells, without any pre-starvation period, significantly responded to exogenous glorin pulses and accumulation of mRNA of model glorin-induced genes was observed within 10 minutes post treatment (Figure 62). This period of time does not appear sufficient enough for *de novo* expression of a regulatory protein or transcription factor that would then differentially regulate the observed target genes.

4.11 Glorin-mediated changes in gene expression do not depend on *de novo* protein expression

Glorin-induced changes in gene expression were not found to be sensitive to cycloheximide treatment confirming that *de novo* protein synthesis is not required to observe glorin effects. Addition of cycloheximide to *P. pallidum* amoebae, immediately after washing away bacterial food, did not affect glorin-mediated gene expression validating that all proteins constituting intracellular glorin signalling cascade are latently present in growing cells (Figure 63). Rather, expression of model glorin-induced genes PPL_09347 and PPL_05833 actually slightly enhanced in cells treated with both cycloheximide and glorin (Figure 63), manifesting a stabilizing effect of cycloheximide on transcripts. Cycloheximide (CHX) inhibits the elongation step of protein synthesis (Vazquez 1979), therefore, in CHX treated cells, ribosomes do not terminate normally and new ribosomes enter the polysome, ultimately saturating the mRNA. Since the stability of mRNA may depend upon the availability of sites for inactivation by nucleases, it seems probable that in the presence of cycloheximide, these sites are protected because in CHX treated cells, the mRNAs are bound to ribosomes that occupy these nuclease sensitive sites, rendering the mRNA stable (Kelly *et al.* 1987). However, starvation-mediated expression of these genes (in the absence of exogenous glorin) was sensitive to cycloheximide treatment (Figure 63), suggesting that induction of these genes in response to starvation may depend on protein expression. In short, these data indicate that the mechanism of glorin-mediated induction of genes pre-exists in growing cells and is distinct from the starvation induction mechanism for these genes.

4.12 Possible molecular mechanisms by which glorin may modulate gene expression changes

In *D. discoideum*, cAMP plays a dual role as it serves both as chemotactic agent to coordinate aggregation of starving amoebae and as secondary messenger to elicit signal transduction pathways. Binding of extracellular cAMP to cAR1 receptor leads to the activation of ACA and ERK2, which together promote a dramatic increase in intracellular cAMP that activates cAMP-dependent protein kinase (PKA). PKA inturn regulates the expression of aggregation-stage genes (Zhang *et al.* 2005; Iranfar *et al.* 2003; Mann *et al.* 1997; Schulkes & Schaap 1995). *Dictyostelium* cAMP dependent protein kinase A (PKA) is a complex composed of an inhibitory regulatory subunit (PKA-R) and a catalytic subunit (PKA-C) (Funamoto *et al.* 2003; Mann *et al.* 1997; Mann *et al.* 1994; Hopper *et*

al. 1993a; Harwood *et al.* 1992a; Mann *et al.* 1992; Burki *et al.* 1991; Firtel & Chapman 1990). The primary target for intracellular cAMP is the regulatory subunit of PKA, which upon cAMP binding dissociates from the catalytic subunit that leads to its activation.

In *P. pallidum*, unlike *D. discoideum,* cAMP is not the chemoattractant mediating aggregation. However, occurrence of a developmentally regulated cAMP-dependent protein kinase has been reported in *P. pallidum* that is similar in its properties with PKA isolated from *D. discoideum* (Francis *et al.* 1984). Funamoto and colleagues (Funamoto *et al.* 2003) showed that *Dictyostelium* PKA subunits introduced into *Polysphondylium* amoebae were functional as signal components demonstrating that a biochemically similar PKA signalling pathway works in *Polysphondylium*. The authors showed that *P. pallidum* cells overexpressing dominant negative mutant of the regulatory subunit (that is unable to bind cAMP) from *Dictyostelium*, did not form typical aggregation streams, rather many small clumps were formed that eventually differentiated to small mounds and finally very small fruiting bodies were formed without branching of secondary sorogens (Funamoto *et al.* 2003). *P. pallidum* PN500 cells overexpressing the catalytic subunit (PKA-C) of *Dictyostelium* developed precociously and rapidly formed many small sized mounds. In *P. pallidum,* there is developementally regulated increase in the activity of cAMP-dependent protein kinase (PKA) before aggregates are formed (Funamoto *et al.* 2003) indicating that PKA may be required for the aggregation of *P. pallidum*.

D. discoideum ACA (Adenylyl cyclase A) is a development-specific membrance-bound enzyme activated by extracellular cAMP through cARs to synthesize cAMP in the aggregation process. Most of the synthesized cAMP is secreted to coordinate cell-cell communication, however, some cAMP is retained intracellularly and activates PKA. *D. discoideum*, *acaA* null mutants (*acaA*$^{-}$*)* exhibit defective chemotactic cAMP signal relay and inefficient aggregation (Galardi-Castilla *et al.* 2010; Alvarez-Curto *et al.* 2001; Pitt *et al.* 1993; Pitt *et al.* 1992). Glorin is supposed to be the acrasin of *P. pallidum*, therefore, normal aggregation in this species is assumed to require glorin-induced changes in gene expression. Recently, Schaap and colleagues (personal communication) showed that *P. pallidum* PN500 mutants lacking two putative aggregation-adenylyl cyclases were still able to aggregate, thereby demonstrating that increases in internal levels of cAMP may not be required to activate PKA for glorin-induced changes in gene expression. Alternatively, if intracellular cAMP regulates gene expression by triggering PKA activity, then constitutive PKA activity should be sufficient for full gene expression independent of

cAMP synthesis. PKA-C is inhibited when associated with its regulatory subunit but gets activated when intracellular cAMP binds to the PKA-R. It was shown that in *Dictyostelium* the PKA-R and PKA-C subunits are only loosely connected (Pitt *et al.* 1993; De Gunzburg *et al.* 1984) and when PKA-C outnumbers the regulatory subunit, some of the constitutive and intracellular cAMP-independent PKA activity is speculated (Schaap 2011b; Pitt *et al.* 1993; Anjard *et al.* 1992). Another possibility is that *P. pallidum* PKA-C may be acutely sensitive to a cell surface glorin receptor-mediated intracellular signalling cascade and cells can produce an excess of C over R. Alternatively, PKA may be activated by agents other than cAMP (Pitt *et al.* 1993). Another explanation could be that cGMP induces some PKA activation because basal cGMP may be present at higher levels than cAMP (Pitt *et al.* 1993). In mammalian cells, it has been shown that cGMP regulates gene expression both at transcriptional and posttranscriptional levels (Pilz & Casteel 2003). Moreover, in *P. pallidum* mutant cells lacking two potential *aca* genes, glorin-stimulation mediated increases in cGMP accumulation may be normal, leading to regular changes in gene expression. However, the presence of a third adenylyl cyclase enzyme is suspected in *P. pallidum* PN500 that may be responsible to provide intracellular cAMP for activation of PKA in the absence of other two ACA enzymes (Pauline Schaap; Personal communication).

Furthermore, it was shown that in *P. pallidum* there is a PKA-dependent and a non-PKA-dependent intracellular signalling pathway regulating early developmental genes (Funamoto *et al.* 2003). Additionally, results presented in this thesis showed that exogenous glorin pulses have no influence on *pka*C expression (Figure 58). However, expression of *erkB* (PPL_12271; also known as *erk2*) was enhanced by glorin treatment (Figure 26). In *D. discoideum*, PKA-requiring cAMP-pulse-induced gene (cPIG) expression requires ERK2, owing to the ability of ERK2 to inhibit RegA. However, ERK2 is also required for induction of non-PKA-requiring cPIG expression in *D. discoideum*, indicating that ERK2 regulates PKA-independent pulse-induced genes by a distinct mechanism. It is possible that in *P. pallidum* ERK2 may govern glorin-regulated gene expression by the direct phosphorylation and activation of a transcription factor or a nuclear regulatory protein; a role exhibited by MAP kinases in other systems ranging from yeasts to mammals (Whitmarsh & Davis 1999). Knetsch and colleagues (Knetsch *et al.* 1996) showed that in *D. discoideum* roughly 1-2% of ERK2 translocates to the nucleus upon cAMP stimulation; however, a direct role of ERK2 in transcriptional regulation still remains to be established. Besides RegA, another putative substrate of ERK2 is EppA that is required for chemotaxis and cAMP production during early

developmental stages in *D. discoideum* (Hadwiger & Nguyen 2011; Chen & Segall 2006). Nevertheless, EppA does not seem to serve as transcription factor for PKA-independent gene expression because of its cytosolic localization and absence of known DNA binding motifs. *D. discoideum erkB*$^-$ cells are impaired in development and fail to aggregate (Segall *et al.* 1995), possibly due to the failure to induce PKA-independent cAMP pulse-induced genes, which are critical for cAMP signalling.

A clear understanding of whether or not glorin induces changes in gene expression through a pathway independent of PKA activation, would require studying the gene expression changes in response to exogenous glorin in various mutants altered in PKA signalling, i.e. *P. pallidum* PN500 cells overexpressing PKA-C and PKA-Rm, cells lacking both aggregation-stage adenylyl cyclases, and the MAP kinase ERK2 (erkB$^-$) that exhibit lack of intracellular cAMP and PKA activity.

4.13 Differences between glorin-signalling system and cAMP acrasin system of *D. discoideum*

Although the glorin signalling system shares many parallels with the cAMP acrasin system of *D. discoideum*, there are also considerable differences. The cAMP signalling system is developmentally regulated in *D. discoideum* cells and cascades of protein synthesis are required to induce most of cAMP-dependent genes. The results presented in this dissertation suggest that in *P. pallidum* cells all basic elements of glorin-signalling are latently present in growing cells. Studies of the glorin signalling system in *P. violaceum* cells revealed that amoebae possess a considerable number of glorin receptors during the growth phase with only a slight increase during early aggregation (De Wit *et al.* 1988). Furthermore, *D. discoideum* cells secrete cAMP to the extracellular space in response to a cAMP stimulus, thereby relaying the signal into the surrounding region that causes amoebae to aggregate in stream pattern. Conversely, glorin-induced glorin secretion has not been detected in aggregating *P. violaceum* cells (De Wit *et al.* 1988), however, *P. violaceum* or *P. pallidum* amoebae join together to form moving streams of cells that converge at aggregation centres. Nevertheless, it still remains to be answered whether aggregation of *P. violaceum* or *P. pallidum* amoebae involves any kind of signal relay. It can not be ruled out that glorin stimulus may induce the export of a signal molecule other than glorin itself to propagate signal in neighbouring cells. Previous studies reported developmentally regulated, glorin-induced cGMP accumulation in *P. violaceum* cells that reached to its maximum during aggregation but declined at the onset

of culmination (De Wit *et al.* 1988). Interestingly, it has been shown that cGMP, the secondary messenger, is vigorously exported from many mammalian cell types in quantities that exceed that of cAMP (Hofer & Lefkimmiatis 2007; Andric *et al.* 2006; Sager 2004). So far, in mammalian cells, specific molecular receptors for cGMP have not yet been identified. However, in *D. discoideum* cells, at concentration of 10^{-4} M cGMP binds to the aggregation stage cAMP receptor (Mato *et al.* 1978). Later, Klein *et al.* (1985) mentioned that cyclic GMP can also behave as chemoattractant for *D. discoideum* cells at high concentrations and could trigger cell differentiation to aggregation competence similar to extracellular cAMP (Klein *et al.* 1985). In another study, when *D. discoideum* and *D. lacteum* cells were stimulated with their respective chemoattractants, 20% of the intracellularly accumulated cGMP was secreted (Van Haastert *et al.* 1983). Several biological roles of extracellular cGMP have been described for mammalian cells (Hofer & Lefkimmiatis 2007; Sager 2004). However, in *Dictyostelids*, putative roles of extracellular cGMP in transferring information to neighbouring cells have not yet been identified.

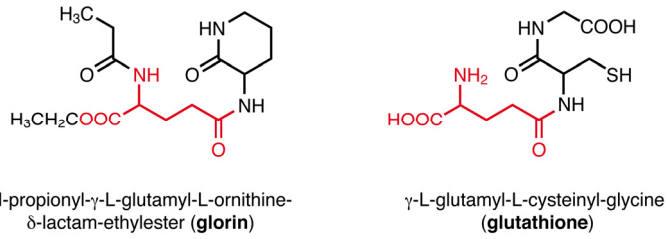
Moreover, it can not be ignored that 'relay' may be required to propagate the signal extensively into the field of starving cells leading to aggregation of more cells at central collection points that may favour bigger aggregate size and therefore formation of the larger fruiting bodies that are characteristic of group 4 species. However, the formation of smaller fruiting bodies typical for group 2 species such as *P. pallidum* may be acquired without any signal relay.

4.14 How did the glorin acrasin system evolve?

A few hypotheses can be presented regarding evolution of glorin acrasin system. First attractive idea is that glorin may be secreted by some kind of bacteria and amoebae invented cell-surface receptors to detect glorin-producing bacteria as food. In the course of evolution, amoebae converted this food-seeking mechanism into an acrasin system to coordinate their own aggregation by inventing few more proteins. However, this assumption is confronted by the consideration that glorin appears to be a product of secondary metabolism, and then it seems rather pointless that social amoebae will adapt food-seeking system into an acrasin system because then amoebae would have required the invention of an entirely new biosynthetic machinery to synthesize glorin. A second possibility is that all acrasin system genes are closely linked on a chromosome in bacteria or some other soil microorganism, and that there has been a horizontal transfer

of a segment of DNA containing this entire genetic material from bacteria to amoebae. It is now known that such transfers events are entirely possible and in *D. discoideum* 16 genes have been recognized as horizontal gene transfer (HGT) events (Sucgang *et al.* 2011).

Contrarily, Bonner (1982) hypothesized that an acrasin can be any small, readily diffusible molecule regularly synthesized and degraded in the cell, and with a receptor and other associated proteins already present (Bonner 1982). This idea seems quite reasonable because known acrasin systems in *Dictyostelids*, using cAMP, folic acid or pterins as aggregation mediators, employ existing cellular biochemical machinery to exploit these primary metabolites as acrasins. Interestingly, chemical structure of glorin shares some similarities with that of glutathione (GSH) as shown in Figure 66.



N-propionyl-γ-L-glutamyl-L-ornithine-δ-lactam-ethylester (**glorin**)

γ-L-glutamyl-L-cysteinyl-glycine (**glutathione**)

Figure 66: Chemical structure of glorin (left) and glutathione (right). Glorin is a dipeptide of glutamate and ornithine, in which amino group and a carboxyl group of the glutamate are blocked by a propionyl group and an ethyl ester, respectively. An amino group in the ornithine moiety is blocked by the formation of a lactam ring.

Glutathione is a ubiquitous tripeptide with an unusual peptide linkage between the amine group of cysteine (which is attached by normal peptide linkage to a glycine) and the carboxyl group of the glutamate side-chain. It plays critical roles in antioxidant defense, regulation of cellular events including gene expression, nutrient metabolism, DNA and protein synthesis, amino acid transport, enzyme activation, cell proliferation, apoptosis, and signal transduction (Kim *et al.* 2005; Wu *et al.* 2004). The glutathione biosynthetic machinery is found in some bacteria including cyanobacteria and proteobacteria (*E.coli, Salmonella*, *Vibrio*, *Helicobacter*). However, most eukaryotes synthesize glutathione, including *Dictyostelids* and humans. Some previous studies reported that the addition of glutathione to *Amoeba proteus* elicits pseudopod formation, activates chemotactic movement towards food source and promotes phagocytosis (Prusch & Minek 1985) suggesting that glutathione-related substances released by certain bacteria or other

microorganisms may act as chemical cues that help amoebae to recognize and locate a suitable prey organism.

In the cells of central nervous system, glutathione acting as a neuromodulator exerts its action by binding to glutamate receptors. Interestingly, several putative metabotropic glutamate receptors are encoded by *P. pallidum* PN500 genome (Heidel *et al.* 2011). Moreover, homologs of all genes encoding components of glutathione biosynthesis and degradation appear to be present in *P. pallidum* PN500 cells (Social Amoebas Comparative Genome Browser: http://sacgb.fli-leibniz.de/cgi/index.pl). Recently, it has been shown that glutathione is essential for *Dictyostelium* growth and development (Kim *et al.* 2005). It seems probable that glutathione is accumulated in *Polysphondylium* cells during the stress response and a suspected correlation between glutathione metabolism and glorin synthesis can not be entirely excluded. This hypothesis is supported by the fact that developmental cAMP signalling in the *Dictyostelids* has also evolved from stress response (Ritchie *et al.* 2008) and favours the concept that opportunistic exploitation of existing genes is a major phenomenon in the emergence of novel characters (Kawabe *et al.* 2012). If this is the case, then all the major components of an acrasin system seem to be encoded in the genome, and already present in the cell. Whether structural similarity between glorin and glutathione offers some meanings in the context of molecular functions of these two substances needs to be carefully investigated.

Nevertheless, previous studies showing that growing *Polysphondylium* amoebae possess almost as many glorin receptors on their cell surface as do aggregating cells (Kopachik 1990; De Wit *et al.* 1988) and the results presented in this dissertation provide a basis to assume that glorin may be a signal molecule used by ancient *Dictyostelids* not only to find food but also to coordinate the process of cell aggregation. Thus far, folic acid and pterins were recognized as chemoattractants with dual functions i.e., food detection and organization of aggregation (De Wit & Konijn 1983; Van Haastert *et al.* 1982; Pan *et al.* 1975). The fact that food seeking and cell aggregation make use of same or related signalling molecules may illustrate an evolutionary connection between these two processes.

4.15 Synthesis, storage, and secretion of glorin

A recent review by Hook *et al.* (2010) discusses function of brain neuropeptides in mediating chemical cell–cell communications among neurons in the central nervous

system (Hook *et al.* 2010). Nascent precursors of these peptide transmitters, known as proneuropeptides, are packaged within the newly formed secretory vesicles in the cell body. Proteolytic processing of the precursor protein occurs during axonal transport and maturation of the secretory vesicle (Hook *et al.* 2008). Mature processed neuropeptides are stored within secretory vesicles at the synapse where activity-dependent, regulated secretion of peptide transmitter occurs to mediate neurotransmission when peptidergic receptors are activated by neuropeptides (Hook *et al.* 2010). It may not be presumptuous to hypothesize that synthesis, storage and secretion of glorin in *P. pallidum* may follow a similar mechanism because glorin is an unusual peptide and its synthesis may involve complex biosynthetic steps. Moreover, it does not seem realistic that cells might have to synthesize and secrete glorin simultaneously; as soon as the process of aggregation begins. Furthermore, results presented in this dissertation suggest that all components of glorin signalling are present in the growing cells, therefore, it is reasonable to speculate that in *P. pallidum* cells, glorin is constitutively synthesized and accumulated into 'vacuolar membrane vesicles' or 'secretory granules'. Subsequently, at the onset of aggregation, intracellularly compartmented glorin is eliminated by exocytosis via vesicular secretory pathway. Similar 'storage-excretion mechanisms' are used by mammalian and yeast cells to eliminate a broad range of lipophilic toxins from the cytosol after their conjugation with glutathione (Li *et al.* 1996).

4.16 Perspectives

4.16.1 Determination of mechanisms regulating glorin-induced gene expression

4.16.1.1 Does glorin stimulate *de novo* transcription?

To investigate whether the stimulation of gene expression by glorin pulses is at the transcriptional level, following 3 approaches are suggested:

1. A combination of **actinomycin D** and **daunomycin** rapidly inhibits synthesis of mRNA in *D. discoideum* (Firtel *et al.* 1973). Same drugs may be tested in *P. pallidum* PN500 to examine whether *de novo* transcription is responsible for the rapid increase in transcript abundance in response to glorin treatment.

2. ***In vitro Nuclear run-on assays*****:** *P. pallidum* PN500 cells can be developed in liquid cultures with or without exogenous glorin pulses for 2 hours, followed by isolation of transcriptionally active nuclei from cells at 0 hr and 2 hr. Nuclear run-on assays will be carried out as described by Nellen *et al.* (1987) involving incubation of standard reaction mixtures containing reaction buffer, each of ATP, GTP, CTP and [α-^{32}P]-UTP, RNAase Guard and isolated nuclei at room temperature for 30 minutes. Labelled RNA can then be collected, denatured at 80^{o}C and placed on ice. Samples of plasmid DNA containing sequences corresponding to respective cDNA probes of representative glorin-induced genes (PPL_09347 or PPL_05833) will be linearized and applied onto Hybond-N nylon membrane using a slot blot apparatus. Pre-hybridization procedure will be followed by hybridization using labelled RNA samples. The membrane will be air dried and exposed to X-ray film. To estimate the relative rate of transcription, autoradiogram can be quantified and values will be normalized to the intensity of the actin band after background subtraction. The intensities of signals obtained will give indication of increase in nuclear transcription. Transcription rate will be compared with the rate of increase in cytoplasmic mRNA levels.

3. ***Alpha-amanitin treatment*****:** Nuclear run-on experiments can be performed in the presence and absence of 50 µM alpha-amanitin (RNA polymerase II inhibitor) to determine whether transcription of glorin-regulated genes is catalyzed by RNA polymerase II.

4.16.1.2 Possible post-transcriptional regulation of glorin-induced gene expression

Signal transduction pathways influence the stability of specific transcripts by rapidly modulating the function of RNA binding proteins that regulate mRNA decay. Possibly, mRNA of many genes that are efficiently induced by glorin are synthesized in the cells from the very beginning of development but these transcripts do not accumulate because they may be very unstable until aggregation process starts. However, glorin signalling may lead to potential stabilisation of these specific transcripts, thereby enhancing their level rapidly.

Polyadenylation of message is one of the post-transcriptional mechanisms that contribute to the stabilization of mRNA. Rapid polyadenylation is reported to occur during *Xenopus* oocyte development as well as during the dorsal ventral patterning of the *Drosophila* embryo (Richter 1999). To test the hypothesis that transcripts may be present

in isolated total RNA but lack poly(A)+ tails and increased mRNA abundance could be a result of rapid polyadenylation, quantitative RT-PCR analysis can be performed on cDNA samples synthesized using oligo-dT (that would not prime cDNA synthesized from non-adenylated transcripts) or random hexamer primers followed by comparison of fold changes between hexamer-primed and oligo-dT-primed cDNAs.

To determine whether ***partial transcripts*** were present in isolated total RNA, primer pairs that would amplify small fragments from either 5' or 3' ends of transcripts of model glorin-induced genes PPL_09347 or PPL_05833 can be used and the differences in fold changes between the 5' or 3' end of any of the two transcripts can be measured.

To examine whether ***mRNA are present as intact messages bound to proteins*** in *P. pallidum* PN500 amoebae, cell-free lysates can be incubated with different proteases such as trypsin, proteinase K, or Qiagen protease prior to protein precipitation during RNA extraction. Subsequently, the release of transcripts by protease treatment can be detected by real-time RT-PCR.

In short, an insight into the mechanisms by which glorin regulates gene expression changes may provide an understanding of the modulation of gene expression by other chemotactic facors in eukaryotes.

4.16.2 Identification of glorin stimulus-inducible promoters by ChiP-on-chip

Recently it was reported that kinases (including PKA and ERKs) are physically associated with promoters they regulate (Pokholok *et al.* 2006). Glorin-induced gene expression may be regulated directly by ERK2 or PKA or both. Therefore, ChiP-on-chip technique that combines chromatin immunoprecipitation (*"ChIP"*) with microarray technology (*"chip"*), using antibodies against ERK2 and PKA may indicate a set of glorin-stimulus induced promoters. If the available antibodies are not compatible with chromatin immunoprecipitation, tagged PKA and ERK2 would be an appropriate alternative. The resulting group of similarly regulated promoters can then be used to recognize critical DNA binding elements and may facilitate to determine the factors that regulate the glorin-stimulus inducible promoters.

4.16.3 Identification of *cis*-acting DNA elements that may be responsive to glorin signalling in the promoter region of glorin-regulated genes

To understand molecular basis for the developmental regulation of early gene expression by glorin, it would be interesting to analyze promoter of a model glorin-induced gene such as PPL_09347. For this purpose, reporter-gene constructs will be prepared by fusing promoter (~1000bp-1500bp upstream of transcription start site) of PPL_09347 with a transcription unit that can express a reporter protein such as luciferase. This strategy will help to determine whether this promoter region can regulate the expression of a reporter gene in the same manner as it does for the endogenous gene (i.e. PPL_09347) in response to glorin stimulation. If reporter gene responds to glorin signal in a similar way, it will indicate that this promoter fragment contains glorin response activities and can be used to further define glorin responsive elements. To determine the major glorin response element within the PPL_09347 prototype promoter region, sequential deletions will be made in this region using either PCR amplification or existing restriction enzyme sites. The deletion constructs containing 5'-flanking sequences of PPL_09347 starting at different positions will be cloned into expression vectors and used to transform *P. pallidum* PN500 cells. Transformants will be selected, pooled and assayed for luciferase activity in response to glorin pulsing. Other techniques that can be used to analyze promoter regions include replacing the full length promoter of PPL_09347 with truncations and site-directed mutations. These approaches will help to identify a 'sequence element' within promoter region or transcriptional enhancer region upstream of the PPL_09347 promoter that may be necessary for glorin-induced gene expression. Once recognized, the consensus sequence will be searched in the promoters of other glorin-regulated genes.

4.16.4 Identification of *trans*-acting factors interacting with promoters of glorin-induced genes

The largest changes in transcription are intervened by variations in the levels or activities of transcription factors. Some transcription factors have been identified in *D. discoideum* (Williams 2006; Eichinger *et al.* 2005) that are required for the cells to progress through development such as CbfA (C-module binding factor A; necessary for aggregation; Winckler *et al.* 2004), G-box Binding Factor (GBF; necessary for post-aggregation development due to its critical role in expression of post-aggregative genes; Schnitzler *et al.* 1994), Myb proteins (that play important role at culmination; Tsujioka *et al.* 2007) and

DNG-1. However, transcription factors directly involved in cAMP-pulse induced gene expression in *D. discoideum* have not yet been recognized. Also, no PKA-activated transcription factor has been identified that could be responsible for cAMP pulse-induced PKA-dependent gene expression. Therefore, investigation of tran-acting transcription factors and their specific cis-acting binding sites is of utmost importance for understanding glorin-modulated developmental responses in *P. pallidum*.

In order to understand whether a transcription factor is involved in glorin-regulated gene expression, promoter regions (upstream of the transcriptional start site) of 25 *P. pallidum* PN500 genes, highly upregulated by glorin, were searched to identify a specific nucleotide sequence element common to all these genes where a sequence-specific glorin-activated transcription factor could bind. Surprisingly, no obvious sequence motif was found that was common to all genes. Some C-rich boxes were found, but they also appeared in genes not regulated by glorin, indicating that glorin signalling may not directly activate a transcription factor to regulate gene expression, rather a nuclear factor(s) could be triggered by glorin signalling. It is likely that a sequence-specific protein interaction with a transcriptional enhancer is involved in the glorin-dependent regulation of gene expression. Nevertheless, chromatin structure and histone modification also exert effects on gene expression.

4.16.4.1 ***Gel mobility shift assays:*** It would be interesting to research for the nuclear protein that may interact with specific sequences within promoter of glorin-regulated genes to modulate glorin-mediated gene expression. Gel mobility shift assays can be performed using the DNA fragment of the 5'-flanking sequence of model glorin-induced gene such as PPL_09347. DNA fragment will be obtained by PCR and labelled for the band shift assay. Nuclear extracts, prepared from *P. pallidum* cells developed for 2 hours in the presence or absence of glorin pulsing will be incubated with the labelled fragements. DNA-protein complexes will be separated by native polyacrylamide gel electrophoresis and mobilities of DNA fragments can be visualized by autoradiography. It would be expected that probes form a specific band with nuclear factor(s) resulting in shifting of the nuclear proteins. Since the PPL_09347 is induced by glorin pulses, it will be of interest to determine whether the shifted nuclear factor is also affected by glorin pulses. Any possible increase in the nuclear factor would suggest that glorin-modulated induction of the PPL_09347 gene could in part be through a direct increase in the amount of the specific *trans* activating factor or through DNA-binding function of such a factor. Identification of any DNA-protein interaction will be followed by affinity purification

of interacting protein from nucleoprotein complexes. Once a specific region in the 5'flanking sequence of selected glorin responsive gene is identified as "putative glorin response element", it would be interesting to create mutations in a few bases and analyze whether nuclear protein has stringent nucleotide sequence requirement for binding. For this purpose, gel mobility shift assays can be carried out using both wild type and mutant forms of "glorin response elements". Future work using DNA methylation interference assays will help to further pinpoint specific bases that are involved in protein binding.

4.16.5 Identification of glorinase and enzyme activity assay

Unexpectedly, proteomics experiments conducted in this research work could not detect any putative 'lactamase' protein (that could open ornithine lactam ring of glorin to degrade the signal) in the extracellular filtrates of *P. pallidum* PN500 cells, suggesting that the experimental strategy should be modified. In order to identify glorinase, an alternative approach should include comparison of proteins secreted by wild type *P. pallidum* PN500 cells with that of glorin-treated cells because glorin may directly or indirectly induce the gene encoding glorinase. Proteins that are observed in the extracellular filtrate of glorin treated cells but not in that of untreated cells may include putative glorinase protein. As a next step, biochemical characterization of the glorinase candidate proteins using synthetic glorin as substrate will provide information about enzyme specificity.

An alternative strategy to identify glorinase may include following steps: (1) fractionation of extracellular proteins secreted by *P. pallidum* PN500 cells by ultrafiltration, (2) Determination of glorin degrading activity of all fractions, (3) Further characterization of highly active fraction to purify "active factor" using chromatographic methods, (4) Concentration of highly active fraction using ultrafiltraion, (5) Fractionation of highly active fraction on a gel filtration column and activity testing, (6) Concentration of active fraction eluted from gel filteration column, using ultrafiltration, (7) Fractionation of active fraction on anion exchange column, (8) Seperation of different proteins in the active fraction eluted from anion exchange column by "analytical hydrophobic interaction exchange column", (9) Fractionation of active fractions obtained from anion exchange column on a native polyacrylamide gel, (10) Further fractionation of active fractions obtained from native polyacrylamide gel on a denaturing SDS polyacrylamide gel to determine N-terminal sequence of proteins, (11) Expression of identified proteins in

bacteria, (12) Glorinase activity testing by incubating putative glorinase protein with different concentrations of glorin, followed by HPLC analysis of degradation products.

4.16.6 Identification cell surface of glorin receptor

Previously, it was reported that glorin act by binding to cell-surface G-protein coupled receptor (De Wit *et al.* 1988). Same authors showed that growing *Polysphondylium* amoebae possess adequate number of glorin receptors that increase slightly in aggregating cells (Kopachik 1990; De Wit *et al.* 1988). Results presented in this thesis point to two putative G-protein coupled receptor genes, i.e. PPL_00902 and PPL_05727 that are expressed in growing cells, whereas their expression levels are moderately enhanced in aggregating cells. In *D. discoideum,* aggregation stage cAR1 receptor is induced by nanomolar cAMP pulses (Mu *et al.* 1998; Firtel 1995; Loomis 1996; Louis *et al.* 1993; Saxe *et al.* 1991a, b; Klein *et al.* 1988, 1987; Kimmel 1987). RNA-seq analysis indicated that both of these genes are induced in response to glorin stimulation. Therefore, these two genes may be potential candidates for glorin receptor and future research should focus on studying their functional roles by generating knock-out mutants in *P. pallidum* PN500. Other approach to identify glorin receptor will make use of 'affinity labelling' technique using a derivative of glorin that can form a covalent linkage once bound to the receptor. Alternatively, REMI mutants of *P. pallidum* can be generated, and FACS (fluorescence activated cell sorting) analysis can be performed to sort out mutants defective in binding fluorescent derivatives of glorin.

4.16.7 Determination of the biological roles played by glorin-regulated genes

Future studies should focus to unravel the functions of glorin-regulated genes by generating knock-out mutations in the genes of interest, followed by determination of the cellular processes which have been disrupted or compromised in such mutants. Products of the genes differentially modulated by glorin may be involved not only in the reception and transduction of extracellular glorin signal but also in the synthesis and degradation of this dipeptide chemoattractant.

Summary

The exclusive life cycle of social amoebae with the frequent shifting between uni- and multicellular stages offers an ideal system to study the complex process of intercellular communication. *Dictyostelids* live as single cells for most of their life time, but under unfavourable conditions they can aggregate to form multicellular fruiting bodies that hold dormant spores. A fundamental requisite for aggregative multicellular development is cell-cell communication. A variety of chemical signals including cAMP, folic acid, pterins and glorin are used by *Dictyostelids* to mediate cell-cell communication in the process of aggregation.

The aggregative chemoattractant glorin (N-propionyl-γ-L-glutamyl-L-ornithine-δ-lactam ethyl ester) was originally isolated from *Polysphondylium violaceum*. In this study, a chemotaxis assay was established to study responses of social amoabae to glorin. Chemotaxis of cells to glorin was found in species from all four major phylogenetic groups of *Dictyostelids*. Previously, some aspects of glorin signalling were studied in the species *P. violaceum*, but the putative role of glorin in mediating changes in gene expression remained unexplored. In this study, effects of glorin as a modulator of gene regulation in the early multicellular development were studied in the species *Polysphondylium pallidum* PN500 whose genome has recently been sequenced and that is chemotactically responsive to glorin. Using Illumina RNA sequencing technology it was shown that glorin mediates rapid changes in gene expression in early development of *P. pallidum*. The optimal glorin concentration and the pulsing frequency required to observe maximal response by *P. pallidum* PN500 was determined to be 1 µM glorin applied at 10-minute intervals. However, glorin effects on gene expression were roughly independent of the glorin concentration applied; only the level of induction was higher at high glorin concentrations used whereas patterns of expression remained unchanged under a variety of pulsing conditions. Glorin-induced genes started to accumulate within 5 minutes of glorin treatment and pre-starvation was not required to detect glorin-induced gene expression, indicating that all basic components of glorin signalling are already present in a latent state in growing amoebae. Developmental kinetics of selected glorin-induced genes showed that most of the glorin-induced genes were maximally expressed at the aggregation stage in the development of *P. pallidum* cells. *P. pallidum* cells treated with glorin exhibited accelerated aggregation when plated for development on agar, most probably because glorin induced precocious expression of aggregation specific genes.

These results imply that glorin is the most ancient intercellular communication molecule that mediates not only chemotactic aggregation but also prepares cells to post-aggregative development by inducing genes required for intra- and intercellular signaling in the multicellular organism. Interestingly, however, the glorin-based communication during aggregation has been replaced several times by other communication systems during dictyostelid evolution.

Zusammenfassung

Der exklusive Lebenszyklus "sozialer Amöben", der häufig zwischen ein- und mehrzelligen Stadien wechselt, bietet ein ideales System für das Studium komplexer Prozesse der interzellulären Kommunikation. Soziale Amöben leben als einzellige Organismen, können aber unter ungünstigen Umweltbedingungen vielzellige Fruchtkörper mit ausdauernden Sporen bilden. Eine Vielzahl chemischer Signale, einschließlich zyklischem AMP, Folsäure, Pterine-Derivaten und Glorin werden von sozialen Amöben verwendet, um die Zell-Zell-Kommunikation während der Aggregation zu vermitteln.

Das chemotaktisch wirkende Molekül Glorin (N-Propionyl-γ-L-glutamyl-L-ornithin-δ-lactam-ethylester) wurde ursprünglich aus *Polysphondylium violaceum* isoliert. In dieser Studie wurde zunächst ein Chemotaxis-Test entwickelt, der es erlaubt, die Reaktion von Zellen auf Glorin zu untersuchen. Es wurde gezeigt, dass Chemotaxis auf Glorin in sozialen Amöben aller vier phylogenetisch verwandter Gruppen der sozialen Amöben vorkommt. Einige Aspekte der Glorin-basierten interzellulären Kommunikation wurden früher in *P. violaceum* studiert, allerdings blieb die mögliche Rolle von Glorin in der Regulation der Genexpression weitgehend unerforscht. In dieser Studie wurden Effekte von Glorin auf die Genexpression in der frühen Entwicklung der Spezies *Polysphondylium pallidum* untersucht, dessen Genom kürzlich sequenziert wurde und die eine chemotaktische Reaktion von Glorin zeigt. Mittels Illumina RNA-Sequenzierung wurde gezeigt, dass Glorin rasche Veränderungen in der Genexpression in der frühen Entwicklung von *P. pallidum* bewirkt. Es wurde gefunden, dass die optimale Konzentration und Pulsfrequenz zur Stimulation der Genexpression bei exogener Zugabe von Glorin zu hungernden Zellen bei 1 µM Glorin und 10-Minuten-Intervallen lag. Der zeitliche Verlauf der Glorin-Effekte auf die Genexpression war allerdings weitestgehend unabhängig von der Glorin-Konzentration. Die Expression Glorin-induzierter Gene wurde bereits innerhalb von fünf Minuten nach Glorin-Zugabe beobachtet und war unabhängig von einer vorherigen Hungerphase der Amöben, was darauf hindeutet, dass das Glorin-basierte Kommunikationssystem schon in wachsenden Zellen latent etabliert ist. Ferner wurde gezeigt, dass die meisten der Glorin-induzierten Gene während der Aggregationsphase in der Entwicklung von *P. pallidum* maximal experimiert werden. Mit Glorin behandelte *P. pallidum* Zellen zeigten eine beschleunigte Aggregation, was möglicherweise als Folge einer verfrühten Expression Glorin-induzierter Gene unter den experimentellen Bedingungen zurückzuführen war.

Die erzielten Ergebnisse lassen vermuten, dass Glorin das evolutionär ältereste interzelluläre Kommunikationsmolekül innerhalb der sozialen Amöben darstellt. Dabei wirkt Glorin nicht nur als chemotaktisches Signalmolekül, sondern konditioniert die Zellen auch für die Postaggragtionsphase, indem es Gene reguliert, die im multizellulären organismus für die intra- und interzelluläre Kommunikation benötigt werden. Bemerkenswerterweise wurde das während der Aggregationsphase aktive Glorin-basierte Kommunikation allerdings in verschiedenen Spezies erfolgreich durch andere Kommunikationssysteme ersetzt.

References

Abe K. and Yanagisawa K. (1983) A new class of rapid developing mutants in *Dictyostelium discoideum*: implications for cyclic AMP metabolism and cell differentiation. *Dev Biol.* **95:**200–210.

Alton T.H. and Lodish H.F. (1977) Translational control of protein synthesis during the early stages of differentiation of the slime mold *Dictyostelium discoideum*. *Cell*. **12(1):**301-10.

Alvarez-Curto E, Meima E. M, Schaap P (2001) Expression and role of adenylyl cyclases during late development in *Dictyostelium discoideum*. *Int. J. Dev. Biol.* **45 (S1)**: S147-S148

Alvarez-Curto E, Rozen D, Ritchie A, Fouquet C, Baldauf SL, Schaap P. (2005) Evolutionary origin of cAMP-based chemoattraction in the social amoebae. *Proc Nat Acad Sci USA.* **102:**6385-6390.

Alvarez-Curto E., Saran S., Meima M., Zobel J., Scott C. & Schaap P. (2007) cAMP production by adenylyl cyclase G induces prespore differentiation in *Dictyostelium* slugs. *Development.* **134:**959–966.

Andric SA, Kostic TS, Stojilkovic SS. (2006) Contribution of multidrug resistance protein MRP5 in control of cyclic guanosine 5'- monophosphate intracellular signaling in anterior pituitary cells. *Endocrinology* 147: 3435–3445.

Anjard C., Pinaud S., Kay R.R. and Reymond C.D. (1992). Overexpression of *Dd*PK2 protein kinase causes rapid development and affects the intracellular cAMP pathway of *Dictyostelium discoideum*. *Development.* **115:**785-790.

Anjard C., Soderbom F., and Loomis W.F. (2001). Requirements for the adenylyl cyclases in the development of *Dictyostelium*. *Development.* **128:** 3649–3654.

Anjard C. & Loomis W.F. (2008) Cytokinins induce sporulation in *Dictyostelium. Development.* **135:**819–827.

Annesley S.J. and Fisher P.R. (2009) *Dictyostelium discoideum*--a model for many reasons. *Mol Cell Biochem*. **329(1-2):**73-91.

Aragon AD, Quiñones GA, Thomas EV, Roy S, Werner-Washburne M. (2006) Release of extraction-resistant mRNA in stationary phase Saccharomyces cerevisiae produces a massive increase in transcript abundance in response to stress. *Genome Biol.* **7(2):**R9 (doi:10.1186/gb-2006-7-2-r9)

Aubry L., Maeda M., Insall R., Devreotes P.N., Firtel R.A. (1997) The *Dictyostelium* mitogen - activated protein kinase ERK2 is regulated by Ras and cAMP-dependent protein kinase (PKA) and mediates PKA function. *J Biol Chem.* **272(7):**3883-6.

Aubry L. & Firtel R.A. (1999). Integration of signaling networds that regulate *Dictyostelium* differentiation. *Annu. Rev. Cell Dev. Biol.* **15:**469-517.

Bagorda A., Das S., Rericha E.C., Chen D., Davidson J., Parent C.A. (2009) Real-time measurements of cAMP production in live *Dictyostelium* cells. *J Cell Sci.* **122(Pt 21):**3907-14.

Baldauf, S. L. & Doolittle, W. F. (1997). Origin and evolution of the slime molds (mycetozoa). *Proc. Natl Acad. Sci. USA.* **94:**12007–12012.

Baldauf, S. L., Roger, A. J., Wenk-Siefert, I. & Doolittle, W. F. (2000). A kingdom-level phylogeny of eukaryotes based on combined protein data. *Science.* **290:**972–977.

Baldauf, S. L. (2003) The deep roots of eukaryotes. *Science* **300:**1703–1706.

Ball J.B, Craik D.J, Alewood P.F, Morrison S, Andrews P.R, Nicholls I.A (1989) Synthesis and conformational analysis of the slime-mould acrasin glorin. *Aust J Chem.* **42**:2171–2180

Barkley D.S. (1969) Adenosine-3',5'-phosphate: identification as acrasin in a species of cellular slime mold. *Science*. **165(3898):**1133-4.

Benabentos R, Hirose S, Sucgang R, Curk T, Katoh M, Ostrowski E.A, Strassmann J.E, Queller D.C, Zupan B, Shaulsky G, Kuspa A. (2009) Polymorphic members of the lag gene family mediate kin discrimination in *Dictyostelium. Curr Biol.* **19(7):**567-72.

Bentley DR, Balasubramanian S, Swerdlow HP, Smith GP, Milton J, Brown CG, Hall KP, Evers DJ, Barnes CL, Bignell HR, Boutell JM, Bryant J, Carter RJ, Keira Cheetham R, Cox AJ, Ellis DJ, Flatbush MR, Gormley NA, Humphray SJ, Irving LJ, Karbelashvili MS, Kirk SM, Li H, Liu X, Maisinger KS, Murray LJ, Obradovic B, Ost T, Parkinson ML, Pratt MR, Rasolonjatovo IM, Reed MT, Rigatti R, Rodighiero C, Ross MT, Sabot A, Sankar SV, Scally A, Schroth GP, Smith ME, Smith VP, Spiridou A, Torrance PE, Tzonev SS, Vermaas EH, Walter K, Wu X, Zhang L, Alam MD, Anastasi C, Aniebo IC, Bailey DM, Bancarz IR, Banerjee S, Barbour SG, Baybayan PA, Benoit VA, Benson KF, Bevis C, Black PJ, Boodhun A, Brennan JS, Bridgham JA, Brown RC, Brown AA, Buermann DH, Bundu AA, Burrows JC, Carter NP, Castillo N, Chiara E Catenazzi M, Chang S, Neil Cooley R, Crake NR, Dada OO, Diakoumakos KD, Dominguez-Fernandez B, Earnshaw DJ, Egbujor UC, Elmore DW, Etchin SS, Ewan MR, Fedurco M, Fraser LJ, Fuentes Fajardo KV, Scott Furey W, George D, Gietzen KJ, Goddard CP, Golda GS, Granieri PA, Green DE, Gustafson DL, Hansen NF, Harnish K, Haudenschild CD, Heyer NI, Hims MM, Ho JT, Horgan AM, Hoschler K, Hurwitz S, Ivanov DV, Johnson MQ, James T, Huw Jones TA, Kang GD, Kerelska TH, Kersey AD, Khrebtukova I, Kindwall AP, Kingsbury Z, Kokko-Gonzales PI, Kumar A, Laurent MA, Lawley CT, Lee SE, Lee X, Liao AK, Loch JA, Lok M, Luo S, Mammen RM, Martin JW, McCauley PG, McNitt P, Mehta P, Moon KW, Mullens JW, Newington T, Ning Z, Ling Ng B, Novo SM, O'Neill MJ, Osborne MA, Osnowski A, Ostadan O, Paraschos LL, Pickering L, Pike AC, Pike AC, Chris Pinkard D, Pliskin DP, Podhasky J, Quijano VJ, Raczy C, Rae VH, Rawlings SR, Chiva Rodriguez A, Roe PM, Rogers J, Rogert Bacigalupo MC, Romanov N, Romieu A, Roth RK, Rourke NJ, Ruediger ST, Rusman E, Sanches-Kuiper RM, Schenker MR, Seoane JM, Shaw RJ, Shiver MK, Short SW, Sizto NL, Sluis JP, Smith MA, Ernest Sohna Sohna J, Spence EJ, Stevens K, Sutton N, Szajkowski L, Tregidgo CL, Turcatti G, Vandevondele S, Verhovsky Y, Virk SM, Wakelin S, Walcott GC, Wang J, Worsley GJ, Yan J, Yau L, Zuerlein M, Rogers J, Mullikin JC, Hurles ME, McCooke NJ, West JS, Oaks FL, Lundberg PL, Klenerman D, Durbin R, Smith AJ. (2008) Accurate whole human genome sequencing using reversible terminator chemistry. *Nature.* **456(7218):**53-9.

Betapudi V, Mason C, Licate L, Egelhoff T.T. (2005) Identification and characterization of a novel alpha-kinase with a von Willebrand factor A-like motif localized to the contractile vacuole and Golgi complex in *Dictyostelium discoideum. Mol Biol Cell.* **16(5):**2248-62.

Bominaar A.A. & Van Haastert P.J. (1994) Phospholipase C in *Dictyostelium discoideum.* Identification of stimulatory and inhibitory surface receptors and G-proteins. *Biochem J.* **297:**189-93.

Bonner J.T. (1947) Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum. J Exp Zool*. **106:**1– 26

Bonner J.T. (1949) The demonstration of acrasin in the later stages of the development of the slime mold Dictyostelium discoideum. *J Exp Zool.* **110(2):**259-71.

Bonner J.T. (1967). *The Cellular Slime Molds.* Princeton University Press.

Bonner J.T, Barkley D.S, Hall EM, Konijn T.M, Mason J.W, O'Keefe G III, Wolfe P.B. (1969) Acrasin, Acrasinase, and the sensitivity to acrasin in *Dictyostelium discoideum*. *Dev Biol.* **20(1):**72-87.

Bonner J.T. (1982) Evolutionary strategies and developmental constraints in the cellular slime molds. *American Naturalist.* **119:**530-552.

Bonner J.T. (1998). The origins of multicellularity. *Integrative Biology*. **1-1**: 27–36.

Bonner JT. (2000). First Signals: The Evolution of Multicellular Development. Princeton, NJ: Princeton Univ. Press. 146 pp.

Bonner, J.T. and Lamont, D. S. (2005). Behavior of cellular slime molds in the soil. *Mycologia.* **97:**178-184.

Bonner J.T. (2009) The Social Amoebae: The Biology of Cellular Slime Molds Princeton University Press, Princeton, N.J.

Bosgraaf L., Russcher H., Smith J.L., Wessels D., Soll D.R., Van Haastert P.J. (2002) A novel cGMP signalling pathway mediating myosin phosphorylation and chemotaxis in *Dictyostelium. EMBO J.* 21(17):4560-70.

Bozzaro S, Gerisch G. (1978) Contact sites in aggregating cells of *Polysphondylium pallidum. J Mol Biol.* **120(2):**265-79.

Braunersreuther V. & Mach F. (2006) Leukocyte recruitment in atherosclerosis: potential targets for therapeutic approaches? *Cell Mol. Life Sci.* **63:**2079–88.

Brazill D.T., Lindsey D.F., Bishop J.D., Gomer R.H. (1998) Cell density sensing mediated by a G protein-coupled receptor activating phospholipase C. *J. Biol. Chem.* **273:**8161-8168.

Brefeld JO. (1869) Dictyostelium mucoroides. Ein neuer organismus aus der Verwandschaft der Myxomiceten. *Abh Senckenb Naturf Ges*. **7:**85-107.

Brenner S, Prösch S, Schenke-Layland K, Riese U, Gausmann U, Platzer C. (2003) cAMP-induced Interleukin-10 promoter activation depends on CCAAT/enhancer-binding protein expression and monocytic differentiation. *J Biol Chem.* 278(8):5597-604.

Brook M, Sully G, Clark A.R, Saklatvala J. (2000) Regulation of tumour necrosis factor alpha mRNA stability by the mitogen-activated protein kinase p38 signalling cascade. *FEBS Lett.* **483(1):**57-61.

Browning D.D, The T, O'Day D.H. (1995) Comparative analysis of chemotaxis in *Dictyostelium* using a radial bioassay method: protein tyrosine kinase activity is required for chemotaxis to folate but not to cAMP. Cell Signal. **7(5):**481-9.

Brzostowski J.A. & Kimmel A.R. (2006) Nonadaptive regulation of ERK2 in Dictyostelium: implications for mechanisms of cAMP relay. *Mol Biol Cell.* **10:**4220-7.

Burki E., Anjard C., Scholder J.C. and Reymond C.D. (1991) Isolation of two genes encoding putative protein kinases regulated during *Dictyostelium discoideum* development. *Gene.* **102:**57–65.

Burns R.A., Livi G.P., Dimond R.L. (1981) Regulation and secretion of early developmentally controlled enzymes during axenic growth in *Dictyostelium discoideum. Dev Biol.* **84(2):**407-16.

Castonguay A.C, Olson L.J, Dahms N.M (2011) Mannose 6-phosphate receptor homology (MRH) domain-containing lectins in the secretory pathway. *Biochim Biophys Acta.* **1810(9):**815-26.

Chang Y.Y. (1968) Cyclic 3',5'-adenosine monophosphate phosphodiesterase produced by the slime mold *Dictyostelium discoideum*. *Science.* **161(3836):**57-9.

Charo I.F. & Taubman M.B. (2004) Chemokines in the pathogenesis of vascular disease. *Circ. Res.* **95:**858–866.

Chassy B.M. (1972) Cyclic nucleotide phosphodiesterase in *Dictyostelium discoideum*: interconversion of two enzyme forms. *Science*. **175(4025):**1016-8.

Chen M.Y., Long Y., Devreotes P.N. (1997). A novel cytosolic regulator, pianissimo, is required for chemoattractant receptor and G protein-mediated activation of the 12 transmembrane domain adenylyl cyclase in *Dictyostelium. Genes Dev.* **11:** 3218-3231.

Chen S. & Segall J.E. (2006) EppA, a putative substrate of DdERK2, regulates cyclic AMP relay and chemotaxis in *Dictyostelium discoideum*. *Eukaryot Cell.* **5:**1136–46.

Chisholm R.L, Hopkinson S, Lodish H.F. (1987) Superinduction of the *Dictyostelium discoideum* cell surface cAMP receptor by pulses of cAMP. *Proc Natl Acad Sci U S A.* **84(4):**1030-4.

Chisholm R.L, Firtel R.A. (2004) Insights into morphogenesis from a simple developmental system. *Nat Rev Mol Cell Biol.* **5:** 531–541.

Chisholm RL, Gaudet P, Just EM, Pilcher KE, Fey P, Merchant SN, Kibbe WA. (2006) dictyBase, the model organism database for *Dictyostelium discoideum. Nucleic Acids Res.* **34(Database issue)**:D423-7.

Clarke M., Yang J., and Kayman S.C. (1988). Analysis of the prestarvation response in growing cells of *Dictyostelium discoideum. Dev. Genet*. **9(4–5):**315–326.

Clarke M., Dominguez N., Yuen I.S., Gomer R.H. (1992). Growing and starving *Dictyostelium* cells produce distinct density-sensing factors. *Dev Biol.* **152(2):**403-6.

Clarke M. and Gomer R.H. (1995) PSF and CMF, autocrine factors that regulate gene expression during growth and early development of *Dictyostelium*. *Experientia* **51:**1124-1134.

Cloonan N., Forrest A.R., Kolle G., Gardiner B.B., Faulkner G.J., Brown M.K., Taylor D.F., Steptoe A.L., Wani S., Bethel G., Robertson A.J., Perkins A.C., Bruce S.J., Lee C.C., Ranade S.S., Peckham H.E., Manning J.M., McKernan K.J., Grimmond S.M. (2008) Stem cell transcriptome profiling via massive-scale mRNA sequencing. *Nat Methods.* **5(7):**613-9.

Clotworthy M. & Traynor D. (2006) On the effects of cycloheximide on cell motility and polarisation in *Dictyostelium discoideum. BMC Cell Biol.* **7:**5.

Cohen M.H. & Robertson A. (1971) Wave propagation in the early stages of aggregation of the cellular slime molds. *J. theor. Biol.* **31:**101-118.

Cohen M.H. & Robertson A. (1972) Differentiation for aggregation in the cellular slime molds. In *Cell Differentiation* (ed. R. Harris, P. Allin & D. Viza), pp. 35-45. Copenhagen: Munksgaard.

Colombatti A, Bonaldo P, Doliana R. (1993) Type A modules: interacting domains found in several non-fibrillar collagens and in other extracellular matrix proteins. *Matrix.* **13(4):**297-306.

Comb M, Birnberg NC, Seasholtz A, Herbert E, Goodman HM. (1986) A cyclic AMP- and phorbol ester-inducible DNA element. *Nature.* **323(6086):**353-6.

Comer F.I., Lippincott C.K., Masbad J.J., Parent C.A. (2005) The PI3K-mediated activation of CRAC independently regulates adenylyl cyclase activation and chemotaxis. *Current Biology.* **15(2):**134-9.

Cosson P, Soldati T. (2008) Eat, kill or die: when amoeba meets bacteria. *Curr Opin Microbiol.* **11(3):**271-6.

Cotter, D. A., Mahadeo, D. C., Cervi, D. N., Kishi, Y., Gale, K., Sands, T., and Sameshima, M. (2000). Environmental regulation of pathways controlling sporulation, dormancy and germination utilizes bacterial like signaling complexes in *Dictyostelium discoideum*. *Protist.* **151(2):**111–126.

Crowley, W. F., Jr., and Hofler J. G., editors. 1987. The Episodic Secretion of Hormones. Churchill Livingstone, New York. 518 pp.

Darmon M, Brachet P, Da Silva L.H. (1975). Chemotactic signals induce cell differentiation in *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. USA* **72:**3163-3166.

Das S, Rericha E.C, Bagorda A, Parent C.A. (2011) Direct biochemical measurements of signal relay during *Dictyostelium* development. *J Biol Chem.* **286(44):**38649-58.

De Cesare D, Sassone-Corsi P. (2000) Transcriptional regulation by cyclic AMP-responsive factors. *Prog Nucleic Acid Res Mol Biol.* **64:**343-69.

De Gunzburg J., Part D., Guiso N., Veron M. (1984) An unusual adenosine cyclic 3',5'-phosphate-dependent protein kinase from *Dictyostelium discoideum*. *Biochemistry*. **23(17):** 3805–3812.

De Wit, R.J.W. & Konijn, T.M. (1983) Identification of the acrasin of Dictyostelium minutum as a derivative of folic acid. *Cell Differ.* **12:**205–210.

De Wit R.J.W., van Bemmelen M.X.P., Penning L.C., Pinas J.E., Calandra T.D., Bonner J.T. (1988) Studies of cell-surface glorin receptors, glorin degradation, and glorin-induced cellular responses during development of *Polysphondylium violaceum*. *Exp Cell Res.* **179:**332–343

de la Roche M.A. & Côté GP. (2001) Regulation of Dictyostelium myosin I and II. *Biochim Biophys Acta.* **1525(3):**245-61.

Desbarats L., Lam T.Y., Wong L.M., Siu C.H. (1992) Identification of a unique cAMP-response element in the gene encoding the cell adhesion molecule gp80 in *Dictyostelium discoideum. J Biol Chem.* **267(27):**19655-64.

Devreotes P.N. (1982). In "The Development of *Dictyostelium discoideum*." (W. F. Loomis, Ed.), pp. 117-168. Academic Press, NY.

Devreotes P.N., Zigmond S.H. (1988) Chemotaxis in eukaryotic cells: a focus on leukocytes and *Dictyostelium. Annu Rev Cell Biol.* **4:**649-86.

Devreotes P.N. (1994) G protein-linked signaling pathways control the developmental program of *Dictyostelium*. *Neuron*.**12:**235–241.

Dormann D. and Weijer C.J. (2003) Chemotactic cell movement during development. *Curr Opin Genet Dev.* **13:** 358-364.

Eccles S.A. (2004) Parallels in invasion and angiogenesis provide pivotal points for therapeutic intervention. *Int. J. Dev. Biol.* **48:**583–598.

Eccles, S.A. (2005) Targeting key steps in metastatic tumour progression. *Curr. Opin. Genet. Dev.* **15:**77–86.

Eichinger L, Pachebat JA, Glöckner G, Rajandream MA, Sucgang R, Berriman M, Song J, Olsen R, Szafranski K, Xu Q, Tunggal B, Kummerfeld S, Madera M, Konfortov BA, Rivero F, Bankier AT, Lehmann R, Hamlin N, Davies R, Gaudet P, Fey P, Pilcher K, Chen G, Saunders D, Sodergren E, Davis P, Kerhornou A, Nie X, Hall N, Anjard C, Hemphill L, Bason N, Farbrother P, Desany B, Just E, Morio T, Rost R, Churcher C, Cooper J, Haydock S, van Driessche N, Cronin A, Goodhead I, Muzny D, Mourier T, Pain A, Lu M, Harper D, Lindsay R, Hauser H, James K, Quiles M, Madan Babu M, Saito T, Buchrieser C, Wardroper A, Felder M, Thangavelu M, Johnson D, Knights A, Loulseged H, Mungall K, Oliver K, Price C, Quail MA, Urushihara H, Hernandez J, Rabbinowitsch E, Steffen D, Sanders M, Ma J, Kohara Y, Sharp S, Simmonds M, Spiegler S, Tivey A, Sugano S, White B, Walker D, Woodward J, Winckler T, Tanaka Y, Shaulsky G, Schleicher M, Weinstock G, Rosenthal A, Cox EC, Chisholm RL, Gibbs R, Loomis WF, Platzer M, Kay RR, Williams J, Dear PH, Noegel AA, Barrell B, Kuspa A. (2005) The genome of the social amoeba *Dictyostelium discoideum*. *Nature.* **5; 435(7038):**43-57.

Ennis H.L., Sussman M. (1958) The initiator cell for slime mold aggregation. *Proc Natl Acad Sci U S A.* **44(5):**401-11.

Escalante R, Vicente J.J. (2000) *Dictyostelium discoideum*: a model system for differentiation and patterning. *Int J Dev Biol.* **44(8):**819-35.

Europe-Finner G.N, Newell P.C. (1985) Inositol 1,4,5-trisphosphate induces cyclic GMP formation in *Dictyostelium discoideum. Biochem Biophys Res Commun.* **130(3):**1115-22.

Europe-Finner G.N, Newell P.C. (1986) Inositol 1,4,5-triphosphate induces calcium release from a non- mitochondrial pool in amoebae of *Dictyostelium. Biochim Biophys Acta.* **887(3):**335-40.

Europe-Finner G.N, Newell P.C. (1987) GTP analogues stimulate inositol trisphosphate formation transiently in *Dictyostelium. J Cell Sci.* **87 (Pt 4):**513-8.

Faure M, Franke J, Hall A.L, Podgorski G.J, Kessin R.H. **(**1990). The cyclic nucleotide phosphodiesterase gene of *Dictyostelium discoideum* contains three promoters specific for growth, aggregation, and late development. *Mol. Cell. Biol.* **10:**1921–1930.

Firtel RA, Baxter L, Lodish HF. (1973) Actinomycin D and the regulation of enzyme biosynthesis during development of *Dictyostelium discoideum. J Mol Biol.* **79(2):**315-27.

Firtel, R.A. and Chapman, A.L. (1990) A role for cAMP-dependent protein kinase A in early *Dictyostelium* development. *Genes Dev.* **4:**18–28.

Firtel R.A. (1995) Integration of signaling information in controlling cell-fate decisions in Dictyostelium. *Genes Dev.* **9(12):**1427-44.

Franca-Koh J, Devreotes P.N. (2004) Moving forward: mechanisms of chemoattractant gradient sensing. *Physiology (Bethesda).* **19:**300-8.

Francis D. (1965) Acrasin and the development of *Polysphondylium pallidum. Dev Biol.* **12(3):**329-46.

Francis D, Majerfeld I.H, Kakinuma S, Leichtling B.H, Rickenberg H.V. (1984) An increase of cAMP-dependent protein kinase during development in *Polysphondylium pallidum. Dev Biol.* **106(2):**478-84.

Franke J., Faure M., Wu L., Hall A.L., Podgorski G.J., and Kessin R.H. (1991). Cyclic nucleotide phosphodiesterase of *Dictyostelium discoideum* and its glycoprotein inhibitor: Structure and expression of their genes. *Dev. Genet.* **12(1–2):**104–112.

Frevel M.A, Bakheet T, Silva A.M, Hissong J.G, Khabar K.S, Williams B.R. (2003) p38 Mitogen-activated protein kinase-dependent and -independent signaling of mRNA stability of AU-rich element-containing transcripts. *Mol Cell Biol.* **23(2):**425-36.

Funamoto S., Milan K., Meili R., Firtel R.A. (2001) Role of phosphatidylinositol 3' kinase and a downstream pleckstrin homology domain-containing protein in controlling chemotaxis in *Dictyostelium*. *J Cell Biol*. **153(4):** 795–810.

Funamoto S, Anjard C, Nellen W, Ochiai H. (2003) cAMP-dependent protein kinase regulates *Polysphondylium pallidum* development. *Differentiation.* 71(1):51-61.

Futrelle R.P, Traut J, McKee W.G. (1982) Cell behavior in *Dictyostelium discoideum*: preaggregation response to localized cyclic AMP pulses*. J Cell Biol.* **92(3):**807-21.

Galardi-Castilla M, Garciandía A, Suarez T, Sastre L. (2010) The *Dictyostelium discoideum acaA* gene is transcribed from alternative promoters during aggregation and multicellular development. *PLoS One.* **5(10):**e13286.

Gaskins C., Clark A.M., Aubry L., Segall J.E., Firtel R.A. (1996) The *Dictyostelium* MAP kinase ERK2 regulates multiple, independent developmental pathways. *Genes Dev.* **10(1):**118-28.

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, et al. (2005) Protein Identification and Analysis Tools on the ExPASy Server. *In: John M. Walker (ed): The Proteomics Protocols Handbook.* Humana Press pp 571- 607.

Gerisch G. (1968). Cell aggregation and differentiation in *Dictyostelium. Curr. Top. dev. Biol.* **3:**l57-l97.

Gerisch G., Malchow D., Riedel V., Muller E., and Every M. (1972) Cyclic AMP phosphodiesterase and its inhibitor in slime mould development. *Nature-New Biol.* **235(55):** 90–92.

Gerisch G., Fromm H., Huesgen A. & Wick U. (1975) Control of cell-contact sites by cyclic AMP pulses in differentiating *Dictyostelium* cells. Nature 255, 547–549.

Gerisch G., Huesgen A., and Malchow D. (1975b). Genetic control of cell differentiation and aggregation in Diotyostelium: The role of cyclic AMP pulses. In “Proceedings of the Tenth FEBS Meeting.”pp. 257-267.

Gerisch G., Malchow D., Roos W., Wick U. (1979). Oscillations of cyclic nucleotide concentrations in relation to the excitability of *Dictyostelium* cells. *J. Exp. Biol*. **81:**33–47.

Gerisch G. (1987) Cyclic AMP and other signals controlling cell development and differentiation in *Dictyostelium*. *Annu Rev Biochem.* **56:**853-79.

Goldbeter A. (2006) Oscillations and waves of cyclic AMP in *Dictyostelium*: a prototype for spatio-temporal organization and pulsatile intercellular communication. *Bull Math Biol.* **68(5):**1095-109.

Goldberg J.M., Manning G., Liu A., Fey P., Pilcher K.E., Xu Y., Smith J.L. (2006) The *dictyostelium* kinome--analysis of the protein kinases from a simple model organism. *PLoS Genet.* **2(3):**e38.

Gomer R.H, Datta S, Mehdy M, Crowley T, Sivertsen A, Nellen W, Reymond C, Mann S, Firtel R.A. (1985) Regulation of cell-type-specific gene expression in *Dictyostelium*. *Cold Spring Harb Symp Quant Biol.* **50:**801-12**.**

Gonzalez-Kristeller D.C, Farage L, Fiorini L.C, Loomis W.F, da Silva A.M. (2008) The P450 oxidoreductase, RedA, controls development beyond the mound stage in *Dictyostelium discoideum. BMC Dev Biol*. **8:**8.

Grant W.N, Williams K.L (1983) Monoclonal antibody characterisation of slime sheath: the extracellular matrix of *Dictyostelium discoideum. EMBO J.* **2(6):**935-40.

Grant CM, Collinson LP, Roe JH, Dawes IW. (1996) Yeast glutathione reductase is required for protection against oxidative stress and is a target gene for yAP-1 transcriptional regulation. *Mol Microbiol.* **21(1):**171-9.

Grosberg R.K., Strathmann R.R. (2007) The Evolution of Multicellularity: A Minor Major Transition? *Annu. Rev. Ecol. Evol. Syst.* **38:**621–54.

Hadwiger J.A. & Nguyen H.N. (2011) MAPKs in development: insights from *Dictyostelium* signaling pathways. *Biomol Concepts.* **2(1-2):**39-46.

Hagiwara H. (1982). Altitudinal distribution of dictyostelid cellular slime molds in the Gosainkund region of Nepal. In: Otani Y., ed. Reports on the cryptogamic study in Nepal. Tokyo: National Science Museum. **p 105–117**.

Hagiwara H. (1989) The Taxonomic Study of Japanese *Dictyostelid* Cellular Slime Molds National Science Museum, Tokyo.

Halloy J, Lauzeral J, Goldbeter A. (1998) Modeling oscillations and waves of cAMP in *Dictyostelium discoideum* cells. *Biophys Chem.* **72(1-2):**9-19.

Hall A.L., Franke J., Faure M., Kessin R.H. (1993). The role of the cyclic nucleotide phosphodiesterase of *Dictyostelium discoideum* during growth, aggregation, and morphogenesis: overexpression and localization studies with the separate promoters of the pde. *Dev. Biol.* **157:** 73-84.

Harwood A.J., Hopper N.A., Simon M.N., Bouzid S., Veron M. and Williams J.G. (1992a) Multiple roles for cAMP-dependent protein kinase during *Dictyostelium* development. *Dev Biol.* **149:**90–99.

Harwood A.J., Hopper N.A., Simon M.N., Driscoll D.M., Veron M. & Williams J.G. (1992b). Culmination in *Dictyostelium* is regulated by the cAMP-dependent protein kinase. *Cell.* **69:** 615–624.

Heidel A.J., Glöckner G. (2008) Mitochondrial genome evolution in the social amoebae. *Mol Biol Evol.* **25(7):**1440-50.

Heidel A.J., Lawal H.M., Felder M., Schilde C., Helps N.R., Tunggal B., Rivero F., John U., Schleicher M., Eichinger L., Platzer M., Noegel A.A., Schaap P., Glöckner G. (2011) Phylogeny-wide analysis of social amoeba genomes highlights ancient origins for complex intercellular communication. *Genome Res.* **21(11):**1882-91.

Hirose S, Benabentos R, Ho H.I, Kuspa A, Shaulsky G. (2011) Self-recognition in social amoebae is mediated by allelic pairs of tiger genes. *Science.* **333(6041):**467-70.

Hofer AM, Lefkimmiatis K. (2007) Extracellular calcium and cAMP: second messengers as "third messengers"? *Physiology* (Bethesda). **22:**320-7.

Hook V, Funkelstein L, Lu D, Bark S, Wegrzyn J, Hwang SR. (2008) Proteases for processing proneuropeptides into peptide neurotransmitters and hormones. *Annu Rev Pharmacol Toxicol.* **48:**393-423.

Hook V, Bark S, Gupta N, Lortie M, Lu WD, Bandeira N, Funkelstein L, Wegrzyn J, O'Connor DT, Pevzner P. (2010) Neuropeptidomic components generated by proteomic functions in secretory vesicles for cell-cell communication. *AAPS J.* **12(4):**635-45.

Hopper N.A., Anjard C., Reymond C.D. and Williams J.G. (1993a) Induction of terminal differentiation of *Dictyostelium* by cAMP-dependent protein kinase and opposing effects of intracellular and extracellular cAMP on stalk cell differentiation. *Development.* **119:**147–154.

Hopper N.A., Harwood A.J., Bouzid S., Ve´ron M. & Williams J.G. (1993b). Activation of the prespore and spore cell pathway of *Dictyostelium* differentiation by cAMP-dependent protein kinase and evidence for its upstream regulation by ammonia. *EMBO J.* **12:**2459–2466.

Insall R., Kay R.R. (1990) A specific DIF binding protein in *Dictyostelium*. *EMBO J.* **9(10):**3323-8.

Insall R., Kuspa A., Lilly P.J., Shaulsky G., Levin L.R., Loomis W.F., Devreotes P. (1994). CRAC, a cytosolic protein containing a pleckstrin homology domain, is required for receptor and G protein-mediated activation of adenylyl cyclase in *Dictyostelium. J. Cell Biol.* **126:**1537-1545.

Insall R.H., Borleis J., Devreotes P.N. (1996). The aimless RasGEF is required for processing of chemotactic signals through G-protein-coupled receptors in *Dictyostelium. Curr. Biol*. **6:** 719-729.

Insall, R.H. (2010). Understanding eukaryotic chemotaxis: a pseudopod-centred view. *Nat. Rev. Mol. Cell Biol.* **11:**453–458.

Iranfar N, Fuller D, Loomis WF. (2003) Genome-wide expression analyses of gene regulation during early development of *Dictyostelium discoideum*. *Eukaryot Cell.* **2(4):**664-70.

Jain R., Gomer R.H. (1994) A developmentally regulated cell surface receptor for a density-sensing factor in *Dictyostelium*. *J Biol Chem.* **269(12):**9128-36.

Janssens P.M, Van Haastert P.J. (1987) Molecular basis of transmembrane signal transduction in *Dictyostelium discoideum*. *Microbiol Rev.* **51(4):**396-418.

Janáky R, Ogita K, Pasqualotto BA, Bains JS, Oja SS, Yoneda Y, Shaw CA. (1999) Glutathione and signal transduction in the mammalian CNS. *J Neurochem.* **73(3):**889-902.

Jia M.H, Larossa R.A, Lee J.M, Rafalski A, Derose E, Gonye G, Xue Z. (2000) Global expression profiling of yeast treated with an inhibitor of amino acid biosynthesis, sulfometuron methyl. *Physiol Genomics.* **3(2):**83-92.

Jin T, Amzel M, Devreotes PN, Wu L. (1998) Selection of gbeta subunits with point mutations that fail to activate specific signaling pathways in vivo: dissecting cellular responses mediated by a heterotrimeric G protein in *Dictyostelium discoideum. Mol Biol Cell.* **9(10):**2949-61.

Jones M.E. (1976) Aggregation in *Polysphondylium. J Cell Sci.* **22(1):**35-40.

Jones M.E, Robertson A. (1976) Cyclic adenosine monophosphate and the development of *Polysphondylium. J Cell Sci.* **22(1):**41-7.

Johnson, R.L., Vaughan R.A., Caterina M.J., Van Haastert P.J. and Devreotes P.N. (1991) Overexpression of the cAMP receptor 1 in growing *Dictyostelium* cells. *Biochemistry*. **30(28:** 6982–6986.

Johnson R.L., Saxe C.L., III Gollop R., Kimmel A.R., and Devreotes P.N.(1993) Identification and targeted gene disruption of cAR3, a cAMP receptor subtype expressed during multicellular stages of *Dictyostelium development. Genes Dev.* **7(2):**273–282.

Juliani M.H, Brusca J, Klein C. (1981) cAMP regulation of cell differentiation in *Dictyostelium discoideum* and the role of the cAMP receptor. *Dev Biol.* **83(1):**114-21.

Jungmann RA, Kelley DC, Miles MF, Milkowski DM. (1983) Cyclic AMP regulation of lactate dehydrogenase. Isoproterenol and N6,O2-dibutyryl cyclic amp increase the rate of transcription and change the stability of lactate dehydrogenase a subunit messenger RNA in rat C6 glioma cells. *J Biol Chem.* **258(8):**5312-8.

Juvan P, Demsar J, Shaulsky G, Zupan B. (2005) GenePath: from mutations to genetic networks and back. *Nucleic Acids Res.* **33(Web Server issue):**W749-52.

Kalla SE, Queller DC, Lasagni A, Strassmann JE. (2011) Kin discrimination and possible cryptic species in the social amoeba *Polysphondylium violaceum*. *BMC Evol Biol.* **11**:31.

Kamboj R.K., Wong L.M., Lam T.Y., Siu C.H. (1988) Mapping of a cell-binding domain in the cell adhesion molecule gp80 of *Dictyostelium discoideum. J Cell Biol.* **107(5):**1835-43.

Kamboj R.K., Gariepy J., Siu C.H. (1989) Identification of an octapeptide involved in homophilic interaction of the cell adhesion molecule gp80 of *Dictyostelium discoideum. Cell.* **59(4):**615-25.

Kawabe Y, Morio T, James J.L, Prescott A.R, Tanaka Y, Schaap P. (2009) Activated cAMP receptors switch encystation into sporulation. *Proc Natl Acad Sci U S A.* **106(17):**7089-94.

Kawabe Y, Weening KE, Marquay-Markiewicz J, Schaap P. (2012) Evolution of self-organisation in Dictyostelia by adaptation of a non-selective phosphodiesterase and a matrix component for regulated cAMP degradation. *Development.*139(7):1336-45.

Kawakami S, Hagiwara H. (2008) *Polysphondylium multicystogenum* sp. nov., a new dictyostelid species from Sierra Leone, West Africa. *Mycologia.* **100(2):**347-51.

Kedersha N, Anderson P. (2002) Stress granules: sites of mRNA triage that regulate mRNA stability and translatability. *Biochem Soc Trans.* **30:**963-969.

Keeling, P. (2004) A brief history of plastids and their hosts. *Protist.* **155:**3–7.

Keeling, P. J., Burger, G., Durnford, D. G., Lang, B. F., Lee, R. W., Pearlman, R. E., Roger, A. J. & Gray, M. W. (2005) The tree of eukaryotes. *Trends. Ecol. Evol.* **20:**670–676.

Kelly R, Shaw DR, Ennis HL. (1987) Role of protein synthesis in decay and accumulation of mRNA during spore germination in the cellular slime mold *Dictyostelium discoideum. Mol Cell Biol.* **7(2):**799-805.

Kessin R.H. (1988) Genetics of early *Dictyostelium discoideum* development. *Microbiol Rev.* **52(1):**29-49.

Kessin R.H. (2001) *Dictyostelium*: evolution, cell biology, and the development of multicellularity Cambridge University Press, Cambridge.

Kessin R.H. (2010) Two different genomes that produce the same result. *Genome Biol.* **11(4):**114.

Kim, H. J., W. T. Chang, M. Meima, J. D. Gross, and P. Schaap. (1998). A novel adenylyl cyclase detected in rapidly developing mutants of *Dictyostelium*. *J. Biol. Chem.* **273:**30859–30862.

Kim BJ, Choi CH, Lee CH, Jeong SY, Kim JS, Kim BY, Yim HS, Kang SO. (2005) Glutathione is required for growth and prespore cell differentiation in *Dictyostelium. Dev Biol.* **284(2):**387-98.

Kimmel A.R, Carlisle B. (1986) A gene expressed in undifferentiated vegetative *Dictyostelium* is repressed by developmental pulses of cAMP and reinduced during dedifferentiation. *Proc Natl Acad Sci U S A.* **83(8):**2506-10.

Kimmel A.R. (1987) Different molecular mechanisms for cAMP regulation of gene expression during *Dictyostelium* development. *Dev Biol.* **122(1):**163-71.

Kimmel A.R. & Parent C.A. (2003) The signal to move: *D. discoideum* go orienteering. *Science*. **300:**1525-1527.

Kimmel A.R., Parent C.A., Gough N.R. (2004) Teaching resources. Spatial and temporal dynamics of signaling components involved in the control of chemotaxis in *Dictyostelium* discoideum. *Sci STKE.* **234:**tr3.

Kimmel AR, Firtel RA. (2004) Breaking symmetries: regulation of *Dictyostelium* development through chemoattractant and morphogen signal-response. *Curr Opin Genet Dev.* **14(5):**540-9.

Kishi, Y., Clements, C., Mahadeo, D. C., Cotter, D. A., and Sameshima, M. (1998) High levels of actin tyrosine phosphorylation: Correlation with the dormant state of *Dictyostelium* spores*. J. Cell Sci.* **111(Pt. 19):**2923–2932.

Klein C, Lubs-Haukeness J, Simons S. (1985) cAMP induces a rapid and reversible modification of the chemotactic receptor in *Dictyostelium discoideum. J Cell Biol.* **100(3):**715-20.

Klein P, Vaughan R, Borleis J, Devreotes P. (1987) The surface cyclic AMP receptor in *Dictyostelium*. Levels of ligand-induced phosphorylation, solubilization, identification of primary transcript, and developmental regulation of expression. *J Biol Chem*. **262(1):**358-64.

Klein P., Theibert A., and Devreotes P. (1988). Identification and ligand induced modification of the cAMP receptor in *Dictyostelium. Methods Enzymol.* **159:**267–278.

Knapinska, A. M., P. Irizarry-Barreto, S. Adusumalli, I. Androulakis, and G. Brewer. (2005). Molecular mechanisms regulating mRNA stability: physiological and pathological significance. *Curr. Genomics.* 6471-486.

Knetsch M.L., Epskamp S.J., Schenk P.W., Wang Y., Segall J.E., Snaar-Jagalska B.E. (1996). Dual role of cAMP and involvement of both G-proteins and ras in regulation of ERK2 in *Dictyostelium discoideum. EMBO J.* **15(13):**3361-8.

Knobil E. (1981). Patterns of hormonal signals and hormone action. *N. Engl. J. Med.* **305:**1582-1583.

Kon T, Adachi H, Sutoh K. (2000) amiB, a novel gene required for the growth/differentiation transition in *Dictyostelium. Genes Cells.* **5(1)**:43-55.

Konijn T.M. (1961) Cell aggregation in *Dictyostelium discoideum.* PhD Thesis. University of Wisconsin. Madison. Wisconsin.

Konijn T.M. Raper K.B. (1961) Cell aggregation in *Dictyostelium discoideum*. *Develop. Biol.* **3:** 725-756.

Konijn T.M, Van De Meene J.G., Bonner J.T., Barkley D.S. (1967) The acrasin activity of adenosine-3',5'-cyclic phosphate. *Proc Natl Acad Sci U S A.* **58(3):**1152-4.

Konijn T.M. (1968) Chemotaxis in the cellular slime molds. II. The effect of cell density. *Biol Bull.* **134(2):**298-304.

Konijn T.M, Barkley D.S., Chang Y.Y., Bonner J.T. (1968) Cyclic AMP: A naturally occurring acrasin in the cellular slime molds. *The American Naturalist.* **102:**225-233.

Konijn T.M., Chang Y.Y., Bonner J.T. (1969) Synthesis of cyclic AMP in *Dictyostelium discoideum* and *Polysphondylium pallidum*. *Nature.* **224(5225):**1211-2.

Konijn T.M. (1969) Effect of bacteria on chemotaxis in the cellular slime molds. *J Bacteriol.* **99(2):**503-9.

Konijn T.M. (1972) Cyclic AMP as a first messenger. *Advances in Cyclic Nucleotide Research.* **1:**17-31

Konijn T. M. (1973) in Proceedings of the Tenth International Congress of Microbiology, Mexico City, Mexico, ed. Perez-Mirauete, A. (Plenum Press, New York), pp. 48-61.

Kopachik W. (1990) Glorin-regulated protein synthesis in *Polysphondylium violaceum*. *Exp Cell Res.* **186:**394–397

Kosaka C. & Pears C.J. (1997) Chemoattractants induce tyrosine phosphorylation of ERK2 in *Dictyostelium discoideum* by diverse signalling pathways. *Biochem. J*. **324(Pt 1):**347–352.

Kumagai A, Pupillo M, Gundersen R, Miake-Lye R, Devreotes P.N., Firtel R.A. (1989) Regulation and function of G-alpha protein subunits in *Dictyostelium. Cell.* **57:** 265-275.

Kumagai A., Hadwiger J.A., Pupillo M., Firtel R.A. (1991) Molecular genetic analysis of two G alpha protein subunits in *Dictyostelium. J Biol Chem.* **266(2):**1220-8.

Lacombe M.L, Podgorski G.J, Franke J, Kessin R.H. (1986) Molecular cloning and developmental expression of the cyclic nucleotide phosphodiesterase gene of *Dictyostelium discoideum. J Biol Chem.* **261(36):**16811-7.

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227(5259):**680-5.

Lalli E., Sassone-Corsi P. (1994) Signal transduction and gene regulation: the nuclear response to cAMP. *J Biol Chem.* **269(26):**17359-62.

Lam L.T, Pickeral O.K, Peng A.C, Rosenwald A, Hurt E.M, Giltnane J.M, Averett L.M, Zhao H, Davis R.E, Sathyamoorthy M, Wahl L.M, Harris E.D, Mikovits J.A, Monks A.P, Hollingshead M.G, Sausville E.A, Staudt L.M. (2001) Genomic-scale measurement of mRNA turnover and the mechanisms of action of the anti-cancer drug flavopiridol. *Genome Biol.* **2(10):**RESEARCH0041.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics.* **23(21):**2947-8.

Larner AC, Jonak G, Cheng YS, Korant B, Knight E, Darnell JE Jr. (1984) Transcriptional induction of two genes in human cells by beta interferon. *Proc Natl Acad Sci U S A.* **81(21):**6733-7.

Lasa M, Brook M, Saklatvala J, Clark A.R. (2001) Dexamethasone destabilizes cyclooxygenase 2 mRNA by inhibiting mitogen-activated protein kinase p38. *Mol Cell Biol.* **21(3):**771-80.

Laub M.T. & Loomis W.F. (1998) A molecular network that produces spontaneous oscillations in excitable cells of *Dictyostelium*. *Mol. Biol. Cell*. **9:**3521–3532.

Lee S., Parent C.A., Insall R., Firtel R.A. (1999). A novel Ras-interacting Protein Required for Chemotaxis and Cyclic Adenosine Monophosphate Signal Relay in *Dictyostelium. Mol. Biol. Cell.* **10:** 2829-2845.

Leng G. (1988). Editor; Pulsatility in Neuroendocrine Systems. *CRC Press, Boca Raton*, Florida. 261 pp.

Li Y, Goldbeter A. (1992) Pulsatile signaling in intercellular communication. Periodic stimuli are more efficient than random or chaotic signals in a model based on receptor desensitization. *Biophys J.* **61(1):**161-71.

Li ZS, Zhao Y, Rea PA. (1995) Magnesium Adenosine 5[prime]-Triphosphate-Energized Transport of Glutathione-S-Conjugates by Plant Vacuolar Membrane Vesicles. *Plant Physiol.* **107(4):**1257-1268.

Li ZS, Szczypka M, Lu YP, Thiele DJ, Rea PA. (1996) The yeast cadmium factor protein (YCF1) is a vacuolar glutathione S-conjugate pump. *J Biol Chem.* **271(11):**6509-17.

Liao X.H, Kimmel A.R. (2009) Biochemical responses to chemoattractants in *Dictyostelium*: ligand-receptor interactions and downstream kinase activation. *Methods Mol Biol.* **571:**271-81.

Lilly P., Wu L., Welker D. L., and Devreotes P.N. (1993). A G-protein beta-subunit is essential for *Dictyostelium* development. *Genes Dev.* **7(6):** 986–995.

Lilly P.J. & Devreotes P.N. (1994) Identification of CRAC, a cytosolic regulator required for guanine nucleotide stimulation of adenylyl cyclase in *Dictyostelium. J Biol Chem.* **269(19):**14123-9.

Lohmann S.M., Vaandrager A.B., Smolenski A., Walter U., and De Jonge H.R. (1997) Distinct and specific functions of cGMP-dependent protein kinases. *Trends Biochem. Sci.* **22:**307–312

Loomis W.F. (1996). Genetic networks that regulate development in *Dictyostelium* cells. *Micro. Rev.* **60:**135-150.

Loomis, W. F., Shaulsky G., and Kuspa A. (1998). Molecular networks that regulate development, p. 201–211. In D. Beysens and G. Forgacs (ed.), Dynamical systems in physics and biology. Springer-Verlag, Paris, France.

Loomis W.F. (1998) Role of PKA in the timing of developmental events in *Dictyostelium* cells. *Microbiol Mol Biol Rev.* **62(3):**684-94.

Loomis W.F. & Shaulsky G. (2011) Developmental changes in transcriptional profiles. *Dev Growth Differ.* **53(4):**567-75.

Louis J.M., Saxe C.L. III, Kimmel A.R. (1993). Two transmembrane signaling mechanisms control expression of the cAMP receptor gene CAR1 during *Dictyostelium* development. *Proc. Natl. Acad. Sci. USA*. **90:**5969–5973.

Ma P.C., Siu C.H. (1990) A pharmacologically distinct cyclic AMP receptor is responsible for the regulation of gp80 expression in *Dictyostelium discoideum. Mol Cell Biol.* **10(7):**3297-306.

Ma H, Gamper M, Parent C, Firtel R.A. (1997) The *Dictyostelium* MAP kinase kinase DdMEK1 regulates chemotaxis and is essential for chemoattractant-mediated activation of guanylyl cyclase. *EMBO J.* **16(14):**4317-32.

MacArthur S, Li X.Y, Li J, Brown J.B, Chu H.C, Zeng L, Grondona B.P, Hechmer A, Simirenko L, Keränen S.V, Knowles D.W, Stapleton M, Bickel P, Biggin M.D, Eisen M.B. (2009) Developmental roles of 21 *Drosophila* transcription factors are determined by quantitative differences in binding to an overlapping set of thousands of genomic regions. *Genome Biol*. **10(7):**R80.

Maeda M, Aubry L, Insall R, Gaskins C, Devreotes P.N., Firtel R.A. (1996). Seven helix chemoattractant receptors transiently stimulate mitogenactivated protein kinase in *Dictyostelium*-Role of heterotrimeric G proteins. *J. Biol. Chem.* **271:**3351–3354.

Maeda M., Firtel R.A. (1997) Activation of the mitogen-activated protein kinase ERK2 by the chemoattractant folic acid in *Dictyostelium. J Biol Chem.* **272(38):**23690-5.

Maeda M., Lu S., Shaulsky G., Miyazaki Y., Kuwayama H., Tanaka Y., Kuspa A., Loomis W.F. (2004) Periodic signaling controlled by an oscillatory circuit that includes protein kinases ERK2 and PKA. *Science.* **304(5672):**875-8.

Mahadeo D.C., Parent C.A. (2006) Signal relay during the life cycle of *Dictyostelium. Current Topics in Developmental Biology*. **73:**115-40.

Mahtani K.R, Brook M, Dean J.L, Sully G, Saklatvala J, Clark A.R. (2001) Mitogen-activated protein kinase p38 controls the expression and posttranslational modification of tristetraprolin, a regulator of tumor necrosis factor alpha mRNA stability. *Mol Cell Biol.* **21(19):**6461-9.

Malkinson A.M, Ashworth J.M. (1973) Adenosine 3':5'-cyclic monophosphate concentrations and phosphodiesterase activities during axenic growth and differentiation of cells of the cellular slime mould *Dictyostelium discoideum. Biochem J.* **134(1):**311-9.

Mangiarotti G, Giorda R, Ceccarelli A, Perlo C. (1985). mRNA stabilization controls the expression of a class of developmentally regulated genes in *Dictyostelium discoideum. Proc Natl Acad Sci U S A.* **82(17):**5786-90**.**

Mann S.K., Firtel R.A. (1987) Cyclic AMP regulation of early gene expression in *Dictyostelium discoideum*: mediation via the cell surface cyclic AMP receptor. *Mol Cell Biol.* **7(1):**458-69.

Mann S.K, Pinko C, Firtel R.A. (1988) cAMP regulation of early gene expression in signal transduction mutants of *Dictyostelium*. *Dev Biol.* **130(1):**294-303.

Mann S.K., Firtel R.A. (1989) Two-phase regulatory pathway controls cAMP receptor-mediated expression of early genes in *Dictyostelium. Proc Natl Acad Sci U S A.* **86(6):**1924-8.

Mann S.K., Firtel R.A. (1991) A developmentally regulated, putative serine/threonine protein kinase is essential for development in *Dictyostelium. Mech Dev.* **35(2):**89-101.

Mann S.K. O., Yonemoto W.M., Taylor S.S. and Firtel R.A. (1992) DdPK3, which plays essential roles during *Dictyostelium* development, encodes the catalytic subunit of cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* **89:**10701-10705.

Mann, S.K.O., Richardson, D.L., Lee, S., Kimmel, A.R. and Firtel, R.A. (1994) Expression of cAMP-dependent protein kinase in prespore cells is sufficient induce spore cell differentiation in *Dictyostelium*. *Proc Natl Acad Sci USA.* **91:**10561–10565.

Mann S.K.O, Brown J.M., Briscoe C., Parent C., Pitt G., Devreotes P. and Firtel R.A. (1997). Role of cAMP-dependent protein kinase in controlling aggregation and post-aggregative development in *Dictyostelium. Dev. Biol*. **183:**208-221.

Martin P., & Parkhurst S.M. (2004) Parallels between tissue repair and embryo morphogenesis. *Development.* **131:**3021–3034.

Martinez MJ, Roy S, Archuletta AB, Wentzell PD, Anna-Arriola SS, Rodriguez AL, Aragon AD, Quiñones GA, Allen C, Werner-Washburne M. (2004) Genomic analysis of stationary-phase and exit in *Saccharomyces cerevisiae*: gene expression and identification of novel essential genes. *Mol Biol Cell.* **15:**5295-305.

Mato JM, Jastorff B, Morr M, Konijn TM. (1978) A model for cyclic AMP-chemoreceptor interaction in *Dictyostelium discoideum. Biochim Biophys Acta.* **544(2):**309-14.

Mayanagi T, Amagai A, Maeda Y. (2005) DNG1, a *Dictyostelium* homologue of tumor suppressor ING1 regulates differentiation of *Dictyostelium* cells. *Cell Mol Life Sci.* **62(15):**1734-43.

McMains V.C., Liao X.H., Kimmel A.R. (2008) Oscillatory signaling and network responses during the development of *Dictyostelium discoideum*. Ageing Res Rev. 234-48. Epub 2008 May 4.

McMullen P.D., Morimoto R.I., Amaral L.A. (2010) Physically grounded approach for estimating gene expression from microarray data. *Proc Natl Acad Sci USA.* **107(31):**13690-5.

Mehdiabadi N.J., Kronforst M.R., Queller D.C., Strassmann JE (2009) Phylogeny, reproductive isolation and kin recognition in the social amoeba, *Dictyostelium purpureum*. *Evolution*. **63(2):**542-548.

Mehdy M.C, Firtel R.A. (1985) A secreted factor and cyclic AMP jointly regulate cell-type-specific gene expression in *Dictyostelium discoideum. Mol Cell Biol.* **5(4):**705-13.

Meili R., Ellsworth C., Firtel R.A. (2000) A novel Akt/PKB-related kinase is essential for morphogenesis in *Dictyostelium. Curr Biol.* **10(12):**708-17.

Meili R., Ellsworth C., Lee S., Reddy T.B., Ma H., Firtel R.A. (1999) Chemoattractant-mediated transient activation and membrane localization of Akt/PKB is required for efficient chemotaxis to cAMP in *Dictyostelium. EMBO J.* **18(8):**2092-105.

Mian I.S, Rose C. (2011) Communication theory and multicellular biology. *Integr Biol* (Camb). **3(4):**350-67.

Milne J.L., Wu L., Caterina M.J., Devreotes P.N. (1995) Seven helix cAMP receptors stimulate Ca2+ entry in the absence of functional G proteins in *Dictyostelium. J. Biol. Chem.* **270:**5926-5931.

Minge MA, Silberman JD, Orr RJ, Cavalier-Smith T, Shalchian-Tabrizi K, Burki F, Skjaeveland A, Jakobsen KS. (2009) Evolutionary position of breviate amoebae and the primary eukaryote divergence. *Proc Biol Sci.* **276(1657):**597-604.

Mohr S, Bakal C, Perrimon N. (2010) Genomic screening with RNAi: results and challenges. *Annu Rev Biochem*. **79:**37-64.

Montminy MR, Sevarino KA, Wagner JA, Mandel G, Goodman RH. (1986) Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc Natl Acad Sci U S A.* **83(18):**6682-6.

Montminy M. (1997) Transcriptional regulation by cyclic AMP. *Annu Rev Biochem.* **66:**807-22.

Mortazavi A, Williams B.A, McClue K, Schaeffer L, Wold B. (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods.* **5:**621–628.

Mrass P. & Weninger W. (2006) Immune cell migration as a means to control immune privilege: lessons from the CNS and tumors. *Immunol Rev.* **213:**195–212.

Mu X, Lee B, Louis J.M, Kimmel A.R. (1998) Sequence-specific protein interaction with a transcriptional enhancer involved in the autoregulated expression of cAMP receptor 1 in *Dictyostelium*. *Development.* **125(18):**3689-98.

Mu X, Spanos SA, Shiloach J, Kimmel A. (2001) CRTF is a novel transcription factor that regulates multiple stages of *Dictyostelium* development. *Development.* **128(13):**2569-79.

Mullens I.A, Franke J, Kappes D.J, Kessin R.H. (1984) Developmental regulation of the cyclic-nucleotide-phosphodiesterase mRNA of *Dictyostelium discoideum*. Analysis by cell-free translation and immunoprecipitation. *Eur J Biochem.* **142(2):**409-15.

Murdoch GH, Potter E, Nicolaisen AK, Evans RM, Rosenfeld MG. (1982a) Epidermal growth factor rapidly stimulates prolactin gene transcription. *Nature.* **300(5888):**192-4.

Murdoch GH, Rosenfeld MG. (1982b) Eukaryotic transcriptional regulation and chromatin-associated protein phosphorylation by cyclic AMP. *Science.* **218(4579):**1315-7.

Murdoch G.H, Franco R, Evans R.M, Rosenfeld M.G. (1983) Polypeptide hormone regulation of gene expression. Thyrotropin-releasing hormone rapidly stimulates both transcription of the prolactin gene and the phosphorylation of a specific nuclear protein. *J Biol Chem.* **258(24):**15329-35.

Myre M.A, Lumsden A.L, Thompson M.N, Wasco W, Macdonald M.E, Gusella J.F. (2011) Deficiency of huntingtin has pleiotropic effects in the social amoeba *Dictyostelium discoideum*. PLoS Genet. 2011 Apr;**7(4):**e1002052. Epub 2011 Apr 28.

Natarajan K, Meyer M.R, Jackson B.M, Slade D, Roberts C, Hinnebusch A.G, Marton M.J. (2001) Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. *Mol Cell Biol.* **21(13):**4347-68.

Nellen W, Datta S, Reymond C, Sivertsen A, Mann S, Crowley T, Firtel RA. (1987) Molecular biology in *Dictyostelium*: tools and applications. *Methods Cell Biol*. **28:**67-100.

Newcomb LL, Diderich JA, Slattery MG, Heideman W. (2003) Glucose regulation of *Saccharomyces cerevisiae* cell cycle genes. *Eukaryot Cell.* **2(1):**143-9.

Newth C.K., Hanna M.H. (1984) Chemotactic response of wild-type and aggregation-defective mutants of *Polysphondylium violaceum. Differentiation*. **28:** 94-100

Noce T, Okamoto K, Takeuchi I. (1983) Purification and characterization of the extracellular cyclic AMP phosphodiesterase of *Dictyostelium discoideum. J Biochem.* **93(1):**37-45.

Noegel A, Gerisch G, Stadler J, Westphal M. (1986). Complete sequence and transcript regulation of a cell adhesion protein from aggregating *Dictyostelium* cells. *EMBO J.* **5(7):**1473-6.

Noegel A.A, Blau-Wasser R, Sultana H, Müller R, Israel L, Schleicher M, Patel H, Weijer C.J. (2004) The cyclase-associated protein CAP as regulator of cell polarity and cAMP signaling in *Dictyostelium. Mol Biol Cell.* **15(2):**934-45.

Nowrousian M. (2010) Next-generation sequencing techniques for eukaryotic microorganisms: sequencing-based solutions to biological problems. *Eukaryot Cell.* **9(9):**1300-10.

Olive E.W (1901) A preliminary enumeration of the sorophoreae. *Proc Am Acad Arts Sci.* **37:**333-344

Olive, E.W. (1902) Monograph of the Acrasieae. Proc. Boston Soc. Nat. Hist. **30:** 451-5l3-.pl.5-8.

Otsuka H, Van Haastert PJ. (1998) A novel Myb homolog initiates *Dictyostelium* development by induction of adenylyl cyclase expression. *Genes Dev.* **12(11):**1738-48.

Palatnik C.M., Storti R.V., Jacobson A. (1979). Fractionation and functional analysis of newly synthesized and decaying messenger RNAs from vegetative cells of *Dictyostelium discoideum. J Mol Biol.* 128(3):371-95.

Palatnik C.M., Storti R.V., Capone A.K., Jacobson A. (1980) Messenger RNA stability in *Dictyostelium discoideum:* does poly(A) have a regulatory role? *J Mol Biol.* **141(2):**99-118.

Palsson E., Lee K.J., Goldstein R.E., Franke J., Kessin R.H., and Cox E. C. (1997). Selection for spiral waves in the social amoebae *Dictyostelium*. *Proc. Natl. Acad. Sci. USA* **94(25):**13719–13723.

Pan P, Hall E.M, Bonner J.T. (1972) Folic acid as second chemotactic substance in the cellular slime moulds. *Nat New Biol.* **237(75):**181-2.

Pan P, Hall E.M, Bonner J.T. (1975) Determination of the active portion of the folic acid molecule in cellular slime mold chemotaxis. *J Bacteriol.* **122(1):**185-91

Parent C.A. & Devreotes P.N. (1996). Molecular genetics of signal transduction in *Dictyostelium*. *Annu. Rev. Biochem.* **65:**411-440.

Parent C. A. and Devreotes P. N. (1999). A cell's sense of direction. *Science.* **284**:765-770.

Parent C.A. (2004**).** Making all the right moves: chemotaxis in neutrophils and *Dictyostelium*. *Curr. Opin. Cell Biol.* **16:**4–13.

Parikh A., Miranda E.R., Katoh-Kurasawa M., Fuller D., Rot G., Zagar L., Curk T., Sucgang R., Chen R., Zupan B., Loomis W.F., Kuspa A., Shaulsky G. (2010) Conserved developmental transcriptomes in evolutionarily divergent species. *Genome Biol.* **11(3):**R35.

Pfaffl MW. (2001) A new mathematical model for relative quantification in real-time RT PCR. *Nucleic Acids Res.* **29(9):**e45.

Pilz RB, Casteel DE. (2003) Regulation of gene expression by cyclic GMP. *Circ Res.* **93(11):**1034-46.

Pitt G.S, Milona N, Borleis J, Lin K.C, Reed R.R, Devreotes P.N. (1992) Structurally distinct and stage-specific adenylyl cyclase genes play different roles in *Dictyostelium* development. *Cell.* **69:** 305–315.

Pitt G.S, Brandt R, Lin K.C, Devreotes P.N, Schaap P. (1993) Extracellular cAMP is sufficient to restore developmental gene expression and morphogenesis in *Dictyostelium* cells lacking the aggregation adenylyl cyclase (ACA). *Genes Dev.* **7(11):**2172-80.

Podgorski G.J., Faure M., Franke J., Kessin R.H. (1988). The cyclic nucleotide phosphodiesterase of *Dictyostelium discoideum*: the structure of the gene and its regulation and role in development. *Dev. Genet.* **9:**267-278.

Pokholok DK, Zeitlinger J, Hannett NM, Reynolds DB, Young RA. (2006) Activated signal transduction kinases frequently occupy target genes. *Science.* **313(5786):**533-6.

Potts G. (1902) Zur Physiologie des Dictyostezium mucoroides. *Flora.* **91:** 281-347.

Primpke G, Iassonidou V, Nellen W, Wetterauer B. (2000) Role of cAMP-dependent protein kinase during growth and early development of *Dictyostelium discoideum. Dev Biol.* **221(1):**101-11.

Preston GM, Billis WM, White BA. (1990) Transcriptional and posttranscriptional regulation of the rat prolactin gene by calcium. *Mol Cell Biol.* **10(2):**442-8.

Prusch R.D and Minek D.R. (1985) Chemical stimulation of phagocytosis in *Amoeba proteus* and the influence of external calcium. *Cell Tissue Res*. **242:**557-564

Radonjic M, Andrau JC, Lijnzaad P, Kemmeren P, Kockelkorn TT, van Leenen D, van Berkum NL, Holstege FC. (2005) Genome-wide analyses reveal RNA polymerase II located upstream of genes poised for rapid response upon *S. cerevisiae* stationary phase exit. *Mol Cell.* **18(2):**171-83.

Raghavan A, Ogilvie R.L., Reilly C., Abelson M.L., Raghavan S., Vasdewani J., Krathwohl M., Bohjanen P.R. (2002) Genome-wide analysis of mRNA decay in resting and activated primary human T lymphocytes. *Nucleic Acids Res.* **30(24):**5529-38.

Raghavan A., Bohjanen P.R. (2004) Microarray-based analyses of mRNA decay in the regulation of mammalian gene expression. *Brief Funct Genomic Proteomic.* **3(2):**112-24.

Raper K.B. (1940). Pseudoplasmodium formation and organization in *Dictyostelium discoideum. jf. Elisha Mitchell scient. Soc.* **56:**241-282.

Raper K.B, Thom C (1941). Interspecies mixtures of Dictyosteliaceae. *Am J Bot.* **28:**69-78

Raper KB. (1956) Factors affecting growth and differentiation in simple slime molds. *Mycologia*. **48:**169-205.

Raper KB. (1984) The dictyostelids Princeton University Press, Princeton.

Rapp PE. (1987) Why are so many biological systems periodic? *Prog Neurobiol.* **29(3):**261–273.

Rathi A, Kayman SC, Clarke M. (1991). Induction of gene expression in *Dictyostelium* by prestarvation factor, a factor secreted by growing cells. *Dev. Genet.* **12:**82 87.

Rathi A. and Clarke M. (1992). Expression of early developmental genes in *Dictyostelium discoideum* is initiated during exponential growth by an autocrine dependent mechanism. *Mech. Devel.* **36:** 173-182.

Reymond C.D., Schaap P., Véron M., Williams J.G. (1995) Dual role of cAMP during *Dictyostelium* development. *Experientia.* **51(12):**1166-74.

Richards, T. A. & Cavalier-Smith, T. (2005) Myosin domain evolution and the primary divergence of eukaryotes. *Nature.* **436:**1113–1118.

Richter JD (1999) Cytoplasmic polyadenylation in development and beyond. *Microbiol Mol Biol Rev.* **63:**446-456.

Riedel V., Gerisch G. (1968) Regulation of extracellular cyclic-AMP-phosphodiesterase activity during development of *Dictyostelium discoideum. Biochem Biophys Res Commun.* **42(1):**119-24.

Riedel V., Malchow D., Gerisch G., and Nagele B. (1972). Cyclic AMP phosphodiesterase interaction with its inhibitor of the slime mold, *Dictyostelium discoideum. Biochem. Biophys. Res. Commun.* **46(1):**279–287.

Riedel V, Gerisch G, Müller E, Beug H. (1973) Defective cyclic adenosine-3', 5'-phosphate-phosphodiesterase regulation in morphogenetic mutants of *Dictyostelium discoideum. J Mol Biol.* **74(4):**573-85.

Rigden D.J, Mello L.V, Galperin M.Y. (2004) The PA14 domain, a conserved all-beta domain in bacterial toxins, enzymes, adhesins and signaling molecules. *Trends Biochem Sci.* **29(7):**335-9.

Ritchie A. V., Van Es S., Fouquet C. & Schaap P. (2008). From drought sensing to developmental control: evolution of cyclic AMP signaling in social amoebas. *Mol. Biol. Evol.* **25:**2109–2118.

Robinson M.D., Oshlack A. (2010) A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biol.* **11(3):**R25.

Rodriguez M, Kim B, Lee N.S, Veeranki S, Kim L. (2008) MPL1, a novel phosphatase with leucine-rich repeats, is essential for proper ERK2 phosphorylation and cell motility. *Eukaryot Cell.* **7(6):**958-66.

Roelofs J, Snippe H, Kleineidam RG, Van Haastert PJ. (2001a) Guanylate cyclase in *Dictyostelium discoideum* with the topology of mammalian adenylate cyclase. *Biochem J.* **354:**697-706.

Roelofs J., Meima M., Schaap P., and Van Haastert P.J.M. (2001b) The *Dictyostelium* homologue of mammalian soluble adenylyl cyclase encodes a guanylyl cyclase. *EMBO J.* **20:** 4341–4348.

Roelofs J., Loovers H.M., Van Haastert P.J. (2001c) GTPgammaS regulation of a 12-transmembrane guanylyl cyclase is retained after mutation to an adenylyl cyclase*. J Biol Chem.* **276(44):**40740-5.

Roelofs J. & Van Haastert P.J. (2002) Characterization of two unusual guanylyl cyclases from *dictyostelium. J Biol Chem.* **277(11):**9167-74.

Romeralo M, Escalante R, Sastre L, Lado C. (2007) Molecular systematics of dictyostelids: 5.8S ribosomal DNA and internal transcribed spacer region analyses. *Eukaryotic Cell.* **6(1):**110-116.

Romeralo M, Spiegel FW, Baldauf S. (2010) A Fully Resolved Phylogeny of the Social Amoebas (Dictyostelia) Based on Combined SSU and ITS rDNA Sequences. *Protist.* **161:**539-548.

Romeralo M, Moya-Laraño J, Lado C. (2011a) Social amoebae: environmental factors influencing their distribution and diversity across south-western Europe. *Microb Ecol.* **61(1):**154-65.

Romeralo M, Cavender JC, Landolt JC, Stephenson SL, Baldauf SL. (2011b) An expanded phylogeny of social amoebas (Dictyostelia) shows increasing diversity and new morphological patterns. *BMC Evolutionary Biology*. **11**:84

Romeralo M, Escalante R, Baldauf SL. (2011c) Evolution and Diversity of Dictyostelid Social Amoebae. *Protist.* http://dx.doi.org/10.1016/j.protis.2011.09.004

Roos W., Nanjundiah V., Malchow D. & Gerisch G. (1975). Amplification of cyclic-AMP signals in aggregating cells of *Dictyostelium discoideum. FEBS Lett.* **53:**139–142.

Rot G, Parikh A, Curk T, Kuspa A, Shaulsky G, Zupan B. (2009) dictyExpress: a *Dictyostelium discoideum* gene expression database with an explorative data analysis web-based interface. *BMC Bioinformatics.* **10:**265.

Sager G. (2004) Cyclic GMP transporters. *Neurochem Int.* 45: 865–873.

Samuel E.W. (1961) Orientation and rate of locomotion of individual amebas in the life cycle of the cellular slime mold *Dictyostelium mucoroides*. **Dev Biol.** 3:317-35

Saran S, Meima M.E, Alvarez-Curto E, Weening K.E, Rozen D.E. Schaap P. (2002) cAMP signaling in *Dictyostelium*. Complexity of cAMP synthesis, degradation and detection. *J Muscle Res Cell Motil.* **23:** 793–802.

Sasaki K, Cripe T.P, Koch S.R, Andreone T.L, Petersen D.D, Beale E.G, Granner D.K. (1984) Multihormonal regulation of phosphoenolpyruvate carboxykinase gene transcription. The dominant role of insulin. *J Biol Chem.* **259(24):**15242-51.

Sasaki A.T., Chun C., Takeda K., Firtel R.A. (2004) Localized Ras signaling at the leading edge regulates PI3K, cell polarity, and directional cell movement. J *Cell Biol.* **167(3):**505-18.

Sassone-Corsi P. (1994) Goals for signal transduction pathways: linking up with transcriptional regulation. *EMBO J.* **13(20):**4717-28.

Sawai, S., Thomason, P. A., and Cox, E. C. (2005). An autoregulatory circuit for long-range self-organization in *Dictyostelium* cell populations. *Nature.* **433:**323–326.

Saxe C.L., Ill Johnson R., Devreotes P.N., and Kimmel A.R. (1991a). Multiple genes for cell surface cAMP receptors in *Dictyostelium discoideum*. *Dev. Genet*. **12(1–2:**6–13.

Saxe C.L., Johnson R.L., Devreotes P.N., and Kimmel A.R. (1991b) Expression of a cAMP receptor gene of *Dictyostelium* and evidence for a multigene family. *Genes Dev.* **5**:1-8.

Schaap P, Winckler T, Nelson M, Alvarez-Curto E, Elgie B, Hagiwara H, Cavender J, Milano-Curto A, Rozen DE, Dingermann T, Mutzel R, Baldauf SL (2006) Molecular phylogeny and evolution of morphology in the social amoebas. *Science*. **314:**661–663

Schaap P. (2007) Evolution of size and pattern in the social amoebas. *Bioessays*. **29(7):** 635–644.

Schaap P. (2011a) Evolutionary crossroads in developmental biology: *Dictyostelium discoideum. Development***. 138(3):**387-96.

Schaap P. (2011b) Evolution of developmental cyclic adenosine monophosphate signaling in the Dictyostelia from an amoebozoan stress response. *Dev Growth Differ.* **53(4):**452-62.

Schatzle J., Rathi A., Clarke M., Cardelli J.A. (1991) Developmental regulation of the alpha-mannosidase gene in *Dictyostelium discoideum*: control is at the level of transcription and is affected by cell density. *Mol Cell Biol.* **11(6):**3339-47.

Schatzle J., Bush J., Cardelli J. (1992) Molecular cloning and characterization of the structural gene coding for the developmentally regulated lysosomal enzyme, alpha-mannosidase, in *Dictyostelium discoideum. J Biol Chem.* **267(6):**4000-7.

Schatzle J., Bush J., Dharmawardhane S., Firtel R.A., Gomer R.H., Cardelli J. (1993) Characterization of the signal transduction pathways and cis-acting DNA sequence responsible for the transcriptional induction during growth and development of the lysosomal alpha-mannosidase gene in *Dictyostelium discoideum*. *J Biol Chem.* **268(26):**19632-9.

Schulkes C. and Schaap P. (1995) cAMP-dependent protein kinase activity is essential for preaggegative gene expression in *Dictyostelium*. *FEBS Lett.* **368:**381- 384.

Schnitzler G.R, Fischer W.H, Firtel R.A. (1994) Cloning and characterization of the G-box binding factor, an essential component of the developmental switch between early and late development in *Dictyostelium. Genes Dev.* **8(4):**502-14.

Schnitzler G.R., Briscoe C, Brown J.M., Firtel R.A. (1995). Serpentine cAMP receptors may act through a G protein-independent pathway to induce postaggregative development in *Dictyostelium. Cell.* **81:** 737-745.

Segall J. E., Kuspa A., Shaulsky G., Ecke M., Maeda M., Gaskins C., Firtel R. A., and Loomis W. F. (1995). A MAP kinase necessary for receptor-mediated activation of adenylyl cyclase in *Dictyostelium. J. Cell Biol.* **128:**405–413.

Segall J. E., Kuspa A., Shaulsky G., Ecke M., Maeda M., Gaskins C., Firtel R. A., and Loomis W. F. (1995). A MAP kinase necessary for receptor-mediated activation of adenylyl cyclase in *Dictyostelium. J. Cell Biol.* **128(3):**405–413.

Shaffer B.M. (1953) Aggregation in cellular slime moulds: in vitro isolation of acrasin. Nature. 1953 May 30;171(4361):975.

Shaffer B.M. (1957a) Aspects of aggregation in cellular slime molds. *Am. Nat.* 91, 19-35.

Shaffer B.M. (1957b) Properties of slime-mould amoebae of significance for aggregation. *Q.Jl microsc. Sci.* **98:**377-392.

Shaffer B.M. (1958) Integration in aggregating slime moulds. *Quart. J. Microscop. Sci.* **99:** 103-121

Shaffer B.M. (1961). The cells founding aggregation centers in the slime mould, *Polysphondylium violaceum. J. Exptl. Biol*. **38:**833-849

Shaffer B.M. (1962). The Acrasina. *Adv. Morphogen.* 2, 109-182.

Shevchenko A., Wilm M., Vorm O., Mann M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem.* **68(5):**850-8.

Shim J, Karin M. (2002) The control of mRNA stability in response to extracellular stimuli. *Mol Cells*. **14(3):**323-31.

Shimomura O., Suthers H.L.B, Bonner J.T. (1982) Chemical identity of the acrasin of the cellular slime mold *Polysphondylium violaceum*. *Proc Natl Acad Sci USA.* **79:**7376–7379

Siegert, F. & Weijer, C. J. (1995). Spiral and concentric waves organize multicellular *Dictyostelium* mounds. *Curr. Biol.* **5:**937–943.

Sies H. (1999) Glutathione and its role in cellular functions. *Free Radic Biol Med.* **27(9-10):**916-21.

Simon M.N., Driscoll D., Mutzel R., Part D., Williams J. & Ve´ron, M. (1989) Overproduction of the regulatory subunit of the cAMP-dependent protein kinase blocks the differentiation of *Dictyostelium discoideum. EMBO J.* **8:**2039–2043.

Simpson, A. G. B. & Roger, A. J. (2004) The real 'kingdoms' of eukaryotes. *Curr. Biol.* **14:**R693–R696.

Sinsimer KS, Gratacós FM, Knapinska AM, Lu J, Krause CD, Wierzbowski AV, Maher LR, Scrudato S, Rivera YM, Gupta S, Turrin DK, De La Cruz MP, Pestka S, Brewer G. (2008) Chaperone Hsp27, a novel subunit of AUF1 protein complexes, functions in AU-rich element-mediated mRNA decay. *Mol Cell Biol.* **28(17):**5223-37.

Siol O., Dingermann T., Winckler T. (2006) The C-module DNA-binding factor mediates expression of the *Dictyostelium* aggregation-specific adenylyl cyclase ACA. *Eukaryot Cell.* **5(4):**658-64.

Siu C.H, Sriskanthadevan S, Wang J, Hou L, Chen G, Xu X, Thomson A, Yang C. (2011) Regulation of spatiotemporal expression of cell-cell adhesion molecules during development of *Dictyostelium discoideum. Dev Growth Differ.* **53(4):**518-27.

Souza G.M., da Silva A.M. and Kuspa A. (1999). Starvation promotes *Dictyostelium* development by relieving PufA inhibition of PKA translation through the YakA kinase pathway. *Development.* **126:**3263-3274.

Souza G.M., Lu S.J. and Kuspa A. (1998). YakA, a protein kinase required for the transition from growth to development in *Dictyostelium*. *Development* **125:**2291-2302.

Sobko A., Ma H., Firtel R.A. (2002) Regulated SUMOylation and ubiquitination of DdMEK1 is required for proper chemotaxis. *Dev Cell.* 2(6):745-56.

Soderbom, F., C. Anjard, N. Iranfar, D. Fuller, and W. F. Loomis. 1999. An adenylyl cyclase that functions during late development of *Dictyostelium*. *Development*. **126:**5463–5471.

Stechmann, A. & Cavalier-Smith, T. (2003) The root of the eukaryote tree pinpointed. *Curr. Biol.* **13:**R665–R666.

Strassmann J.E. (2010) In *Dictyostelium*, the social amoeba.Volume 1. Edited by: Breed MD, Moore J. Oxford: Academic Press;:13-519, Encyclopedia of Animal behavior.

Sucgang, R., G. Chen, W. Liu, R. Lindsay, J. Lu, D. Muzny, G. Shaulsky, W. Loomis, R. Gibbs, and A. Kuspa. (2003). Sequence and structure of the extrachromosomal palindrome encoding the ribosomal RNA genes in *Dictyostelium*. *Nucleic Acids Res.* **31:**2361-2368.

Sucgang R, Kuo A, Tian X, Salerno W, Parikh A, Feasley CL, Dalin E, Tu H, Huang E, Barry K, Lindquist E, Shapiro H, Bruce D, Schmutz J, Salamov A, Fey P, Gaudet P, Anjard C, Babu MM, Basu S, Bushmanova Y, van der Wel H, Katoh-Kurasawa M, Dinh C, Coutinho PM, Saito T, Elias M, Schaap P, Kay RR, Henrissat B, Eichinger L, Rivero F, Putnam NH, West CM, Loomis WF, Chisholm RL, Shaulsky G, Strassmann JE, Queller DC, Kuspa A, Grigoriev IV. (2011) Comparative genomics of the social amoebae *Dictyostelium discoideum* and *Dictyostelium purpureum. Genome Biol.* **12(2):**R20.

Sultan M., Schulz M.H., Richard H., Magen A., Klingenhoff A., Scherf M., Seifert M., Borodina T., Soldatov A., Parkhomchuk D., Schmidt D., O'Keeffe S., Haas S., Vingron M., Lehrach H., Yaspo M.L. (2008) A global view of gene activity and alternative splicing by deep sequencing of the human transcriptome. *Science.* **321(5891):**956-60.

Sun T.J. & Devreotes P.N. (1991). Gene targeting of the aggregation stage cAMP receptor cAR1 in *Dictyostelium. Genes Dev.* **5(4):**572–582.

Swaney K. F., Huang C.H. & Devreotes P.N. (2010). Eukaryotic chemotaxis: a network of signaling pathways controls motility, directional sensing, and polarity. *Annu. Rev. Biophys.* **39:**265–289.

Swanson AR, Spiegel FW, Cavender JC (2002) Taxonomy, slime molds, and the questions we ask. *Mycologia.* **94:**968-979.

Tebo J, Der S, Frevel M, Khabar K.S, Williams B.R, Hamilton T.A. (2003) Heterogeneity in control of mRNA stability by AU-rich elements. *J Biol Chem.* **278(14):**12085-93.

Thompson JD, Higgins DG, Gibson TJ. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice*. Nucleic Acids Res.* **22(22):**4673-80.

Tillinghast H.S. & Newell P.C. (1984). Retention of folate receptors on the cytoskeleton of *Dictyostelium* during development. *FEBS Lett.* **176**:325-330.

Tsujioka M, Zhukovskaya N, Yamada Y, Fukuzawa M, Ross S, Williams J.G. (2007) *Dictyostelium* Myb transcription factors function at culmination as activators of ancillary stalk differentiation. *Eukaryot Cell.* **6(3):**568-70.

Trusolino L., Comoglio P.M. (2002) Scatter-factor and semaphorin receptors: cell signalling for invasive growth. *Nat. Rev. Cancer.* **2:**289–300.

Van Dijken P., Bergsma J.C., Van Haastert P.J. (1997) Phospholipase-C-independent inositol 1,4,5-trisphosphate formation in *Dictyostelium* cells. Activation of a plasma-membrane-bound phosphatase by receptor-stimulated Ca2+ influx. *Eur J Biochem.* **244(1):**113-9.

Van Driel R. (1981) Binding of the chemoattractant folic acid by *Dictyostelium discoideum* cells. *Eur J Biochem.* **115(2):**391-5.

van Es S, Virdy K.J, Pitt G.S, Meima M, Sands T.W, Devreotes P.N, Cotter D.A, Schaap P. (1996) Adenylyl cyclase G, an osmosensor controlling germination of *Dictyostelium* spores. *J Biol Chem.* **271(39):**23623-5.

Van Haastert P. J. M., De Wit R.J., Grijpma Y., Konijn T.M. (1982) Identification of a pterin as the acrasin of the cellular slime mold *Dictyostelium lacteum. Proc Natl Acad Sci U S A.* **79(20):** 6270–6274.

Van Haastert P.J, De Wit R.J, Konijn T.M. (1982b) Antagonists of chemoattractants reveal separate receptors for cAMP, folic acid and pterin in *Dictyostelium*. *Exp Cell Res.* **140(2):**453-6.

Van Haastert P.J, Campagne Van Lookeren M.M; Kesbeke F. (1983) Multiple degradation pathways of chemoattractant mediated cGMP accumulation in *Dictyostelium*. *Biochemica et Biophysica Acta.* **756:**67-71

Van Haastert, P.J.M. & Van der Heijden, P.R. (1983) *J. Cell Biol.* **96:**347–353.

Van Haastert P.J., Bishop J.D., Gomer R.H. (1996). The cell density factor CMF regulates the chemoattractant receptor cAR1 in *Dictyostelium*. *J. Cell Biol.* **134:**1543-1549.

Van Haastert P.J. & Kuwayama H. (1997) cGMP as second messenger during *Dictyostelium* chemotaxis. *FEBS Lett.* **410(1):**25-8.

van Es S, Virdy K.J., Pitt G.S., Meima M., Sands T.W., Devreotes P.N., Cotter D.A., Schaap P. (1996) Adenylyl cyclise G, an osmosensor controlling germination of *Dictyostelium* spores*. J. Biol. Chem.* **271:**23623–23625.

Van Driessche N, Shaw C, Katoh M, Morio T, Sucgang R, Ibarra M, Kuwayama H, Saito T, Urushihara H, Maeda M, Takeuchi I, Ochiai H, Eaton W, Tollett J, Halter J, Kuspa A, Tanaka Y, Shaulsky G. (2002) A transcriptional profile of multicellular development in *Dictyostelium discoideum. Development.* **129(7):**1543-52.

Vazquez, D. 1979. Inhibition of protein biosynthesis, p. 155-159. Springer-Verlag KG, Berlin.

Veltman D.M., Roelofs J., Engel R., Visser A.J., Van Haastert P.J. (2005) Activation of soluble guanylyl cyclase at the leading edge during *Dictyostelium* chemotaxis. *Mol Biol Cell.* **16(2):**976-83.

Veron M., Mutzel R., Lacombe M.L., Simon M.N. and Wallet V. (1988) cAMP-dependent protein kinase from *Dictyostelium discoideum*. *Dev Genet.* **9:**247–258.

Waddell D.R. (1982a) The spatial pattern of aggregation centres in the cellular slime mould. *J Embryol Exp Morphol.* **70:**75-98.

Waddell D.R. (1982b) A predatory slime mould. *Nature.* **298:**464–466

Wang N., Soderbom F., Anjard C., Shaulsky G. & Loomis W. F. (1999) SDF-2 induction of terminal differentiation in *Dictyostelium discoideum* is mediated by the membrane-spanning sensor kinase DhkA. *Mol. Cell. Biol.* **19:**4750–4756.

Wang Y, Liu J, Segall J.E. (1998) MAP kinase function in amoeboid chemotaxis. *J Cell Sci.* **111 (Pt 3):**373-83.

Wang Y. & Segall J.E. (1998) The *Dictyostelium* MAP kinase DdERK2 functions as a cytosolic protein in complexes with its potential substrates in chemotactic signal transduction. *Biochem Biophys Res Commun.* **244(1):**149-55.

Wang Y., Chen C.L., Iijima M. (2011) Signaling mechanisms for chemotaxis. *Dev Growth Differ.* 53(4):495-502.

Wang Z., Gerstein M., Snyder M. (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet.* **10(1):**57-63.

Wasinger V.C., Cordwell S.J., Cerpa-Poljak A., Yan J.X., Gooley A.A., Wilkins M.R., Duncan M.W., Harris R., Williams K.L., Humphery-Smith I. (1995) Progress with gene-product mapping of the Mollicutes: *Mycoplasma genitalium*. *Electrophoresis.* **16(7):**1090-4.

Wedel B., and Garbers D. (2001) The guanylyl cyclase family at Y2K. *Annu. Rev. Physiol.* **63:**215–233.

Weijer C.J. (2004) *Dictyostelium* morphogenesis. *Curr Opin Genet Dev.* **14(4):**392-8.

Weijer CJ. (2009) Collective cell migration in development. *J Cell Sci.* **122(Pt 18):**3215-23.

Whitmarsh A.J, Davis R.J (1999) Signal transduction by MAP kinases: regulation by phosphorylation-dependent switches. *Sci STKE.* **(1):**PE1.

Whittaker C.A, Hynes R.O. (2002) Distribution and evolution of von Willebrand/integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere. *Mol Biol Cell.* **13(10):**3369-87.

Willard S.S. and Devreotes P.N. (2006) Signaling pathways mediating chemotaxis in the social amoeba, *Dictyostelium discoideum. Eur J Cell Biol.* **85(9-10):**897-904.

Williams J.G. (1988) The role of diffusible molecules in regulating the cellular differentiation of *Dictyostelium discoideum. Development.* **103(1):**1-16.

Williams J.G, Ceccarelli A., McRobbie S., Mahbubani H., Kay R.R., Early A., Berks M., Jermyn K.A. (1987) Direct induction of *Dictyostelium* prestalk gene expression by DIF provides evidence that DIF is a morphogen. *Cell.* **49(2):**185-92.

Williams J.G. (1997). Prestalk and stalk cell heterogeneity in *Dictyostelium*. *Dictyostelium*- A model system for cell and developmental biology (Maeda, M., Inouye, K, and Takeuchi, I., eds), Universal Press, Inc., 293-304.

Williams J.G. (2006) Transcriptional regulation of *Dictyostelium* pattern formation. *EMBO Rep.* **7(7):**694-8.

Williams J.G. (2010) *Dictyostelium* finds new roles to model. *Genetics.* **185(3):**717-26.

Williams RS, Boeckeler K, Gräf R, Müller-Taubenberger A, Li Z, Isberg RR, Wessels D, Soll DR, Alexander H, Alexander S. (2006) Towards a molecular understanding of human diseases using *Dictyostelium discoideum*. *Trends Mol Med.* **12(9):**415-24.

Winckler T, Iranfar N, Beck P, Jennes I, Siol O, Baik U, Loomis W.F, Dingermann T. (2004) CbfA, the C-module DNA-binding factor, plays an essential role in the initiation of *Dictyostelium discoideum* development. *Eukaryot Cell.* **3(5):**1349-58.

Winzen R, Kracht M, Ritter B, Wilhelm A, Chen C.Y, Shyu A.B, Müller M, Gaestel M, Resch K, Holtmann H. (1999) The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. *EMBO J*. **18(18):**4969-80.

Wong L.M., Siu C-H. (1986). Cloning of cDNA for the contact site A glycoprotein of *Dictyostelium discoideum*. *Proc Natl Acad Sci U S A.* **83(12):**4248-52.

Wu D. & Lin F. (2011) Modeling cell gradient sensing and migration in competing chemoattractant fields. *PLoS One.* **6(4):**e18805.

Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. (2004) Glutathione metabolism and its implications for health. *J Nutr.* **134(3):**489-92.

Wu L., Valkema R., Van Haastert P.J., and Devreotes P.N. (1995a). The G protein beta subunit is essential for multiple responses to chemoattractants in *Dictyostelium. J. Cell Biol.* **129(6):**1667–1675.

Wu L., Franke J., Blanton R.L., Podgorski G.J., and Kessin R.H. (1995b). The phosphodiesterase secreted by prestalk cells is necessary for *Dictyostelium* morphogenesis. *Dev. Biol*. **167(1):**1–8.

Wurster B., Pan P., Tyan G.G., Bonner J.T. (1976) Preliminary characterization of the acrasin of the cellular slime mold *Polysphondylium violaceum. Proc Natl Acad Sci USA.* **73:**795–799

Wurster B, Bozzaro S, Gerisch G. (1978) Cyclic GMP regulation and responses of *Polysphondylium violaceum* to chemoattractants. *Cell Biol Int Rep.* **2(1):**61-9.

Wurster B, Butz U. (1980) Reversible binding of the chemoattractant folic acid to cells of *Dictyostelium discoideum. Eur J Biochem.* **109(2):**613-8.

Xu Q, Shaulsky G. (2005) GOAT: An R Tool for Analysing Gene Ontologytrade mark Term Enrichment. *Appl Bioinformatics*. **4(4):**281-3.

Yamamoto K.R, Alberts B.M. (1976) Steroid receptors: elements for modulation of eukaryotic transcription. *Annu Rev Biochem.* **45:**721-46.

Yuen I.S., Jain R., Bishop J.D., Lindsey D.F., Deery W.J., Van Haastert P.J., Gomer R.H. (1995). A density-sensing factor regulates signal transduction in *Dictyostelium. J. Cell. Biol.* **129:**1251-1262.

Zhang N., Long Y., and Devreotes P.N. (2001). G**γ** in *Dictyostelium*: Its role in localization of G**βγ** to the membrane is required for chemotaxis in shallow gradients. *Mol. Biol. Cell* **12(10):**3204–3213.

Zhang H., Heid P.J., Wessels D., Daniels K.J., Pham T., Loomis W.F., and Soll D.R. (2003). Constitutively active protein kinase A disrupts motility and chemotaxis in *Dictyostelium discoideum. Eukaryot. Cell.* **2:**62–75.

Zhang M, Goswami M, Hereld D. (2005) Constitutively active G protein-coupled receptor mutants block *Dictyostelium* development. *Mol Biol Cell.* **16(2):**562-72.

Zigmond S.H, Joyce M, Borleis J, Bokoch G.M, Devreotes P.N. (1997) Regulation of actin polymerization in cell-free systems by GTPγS and Cdc42. *J Cell Biol.* **138:**363–374

Appendix

Table A1: Summary of proteins identified in the buffer conditioned by aggregating *P. pallidum* PN500 amoebae. The list was assembled from two independent experiments.

Accession Number	Molecular Weight, kDa	Putative protein function
PPL_09146	214	transmembrane protein
PPL_07850	171	nagA, beta-N-acetylhexosaminidase, glycoside hydrolase family 20 protein, beta-hexosaminidase
PPL_06508	143	hypothetical protein
PPL_03654	137	hypothetical protein
PPL_03794	119	manC, alpha-mannosidase
PPL_01936	113	manA, alpha-mannosidase
PPL_04497	111	plbD, phospholipase B-like protein
PPL_09385	110	unpredicted protein
PPL_02389	109	athl1, acid trehalase-like protein 1
PPL_03165	105	Succinyl-diaminopimelate desuccinylase.
PPL_10946	103	aprA, PhoPQ-activated pathogenicity-related protein
PPL_12272	103	hypothetical protein
PPL_04936	102	psaB, puromycin-sensitive aminopeptidase-like protein, metallopeptidase
PPL_11497	102	discoidin I, A chain
PPL_02349	99	unpredicted protein
PPL_02539	98	cpnE, phospholipid-binding protein, copine E
PPL_12255	96	aco1, putative iron regulatory protein, aconitate hydratase, aconitase
PPL_08085	96	alpha-glucosidase
PPL_01713	92	abpC, gelation factor, actin binding protein, filamin
PPL_07033	90	dpp3-2, dipeptidyl-peptidase III
PPL_04038	89	unpredicted protein
PPL_04711	88	psiB, PA14 domain-containing protein
PPL_02660	86	glycoside hydrolase family 18 protein
PPL_02430	82	pyridoxal phosphate-dependent decarboxylase family

		protein
PPL_05694	78	dcd2A, neutral/alkaline nonlysosomal ceramidase family protein, acid ceramidase
PPL_07741	77	AY055590, peptidase C53 family protein
PPL_08459	76	psiJ, PA14 domain-containing protein, discoidin-inducing complex (DIC) protein
PPL_05069	74	putative alpha-N-acetylgalactosaminidase, glycoside hydrolase family 27 protein
PPL_08886	73	hypothetical protein
PPL_04575	70	glb1, glycoside hydrolase family 35 protein, beta-galactosidase
PPL_00324	69	mcfS, mitochondrial substrate carrier family protein, putative mitochondrial carnitine/acylcarnitine transporter
PPL_00313	68	Beta-amylase activity
PPL_09281	66	putative cellulase, cellulose-binding domain-containing protein, glycoside hydrolase family 5 protein
PPL_11402	65	hypothetical protein
PPL_03772	64	Physaropepsin.
PPL_09152	64	plbA, phospholipase B
PPL_01789	64	plbF, phospholipase B-like protein
PPL_02993	63	unpredicted protein
PPL_08963	62	hypothetical protein
PPL_02984	61	unpredicted protein
PPL_08977	60	hypothetical protein
PPL_02985	59	beta-N-acetylhexosaminidase
PPL_04308	59	probable chitinase
PPL_06169	59	cfaD, counting factor associated protein, cathepsin L-like proteinase, peptidase C1A family protein
PPL_00444	58	P10901 Alpha-L-fucosidase precursor (EC 3.2.1.51) (Alpha-L-fucoside fucohydrolase).
PPL_10565	58	putative cholinesterase, carboxylesterase, type B family protein
PPL_09334	58	hypothetical protein
PPL_03598	57	unpredicted protein

PPL_01061	57	hypothetical protein
PPL_05757	57	peptidase S10 family protein, serine carboxypeptidase
PPL_12550	56	amyA, putative alpha-amylase
PPL_02110	55	beta-xylosidase-like protein, glycoside hydrolase family 39 protein
PPL_09813	54	hypothetical protein
PPL_04364	54	saposin B domain-containing protein
PPL_02907	53	glycosyl hydrolase family chitinase
PPL_09525	52	peptidase S28 family protein
PPL_00337	52	expl9, expansin-like protein
PPL_02779	52	cprA, cysteine proteinase 1
PPL_09452	51	hypothetical protein
PPL_02147	50	pldZ, phospholipase D3
PPL_02789	49	PPL_02789
PPL_02060	49	hypothetical protein
PPL_10948	48	phospholipase D
PPL_00750	48	argE, acetylornithine deacetylase
PPL_02386	46	hypothetical protein
PPL_08292	43	unpredicted protein
PPL_05209	42	ctsD, cathepsin D, preprocathepsin D
PPL_06275	42	cyclophilin-type peptidylprolyl cis-trans isomerase (PPIase)
PPL_08791	42	3-carboxymuconate cyclase
PPL_05734	41	unpredicted protein
PPL_08646	41	sodB, superoxide dismutase
PPL_01576	40	peptidase C1A family protein, papain family cysteine protease, cathepsin Z-like protein
PPL_03302	39	hypothetical protein
PPL_07364	38	chitinase
PPL_10376	37	glycoside hydrolase family 25 protein
PPL_05106	36	gghA, peptidase C26 family protein, gamma-glutamyl hydrolase
PPL_03967	34	ctsZ, cathepsin Z precursor, peptidase C1A family protein, papain family cysteine protease
PPL_03069	33	hypothetical protein

PPL_01142	28	hypothetical protein
PPL_04317	28	tpiA, triose phosphate isomerase, triosephosphate isomerase
PPL_01619	28	CMP/dCMP deaminase, zinc-binding domain-containing protein
PPL_07180	27	dscC-2, discoidin I, C chain and B chain, discoidin I, beta chain, discoidin I, gamma chain
PPL_11278	27	glycoside hydrolase family 25 protein
PPL_07647	26	cad3, putative calcium-dependent cell adhesion molecule-3
PPL_12314	26	P80 protein.
PPL_01003	24	hypothetical protein
PPL_06311	23	unpredicted protein
PPL_02846	23	hypothetical protein
PPL_06421	22	superoxide dismutase
PPL_10113	20	NLP/P60 domain-containing protein
PPL_04524	18	DD7-1, galactose-binding domain-containing protein
PPL_02277	17	gcvH1, glycine cleavage system H-protein
PPL_03282	16	cda, cytidine deaminase
PPL_02795	15	putative phospholipid transfer protein
PPL_04564	15	cyb5A, cytochrome b5 A
PPL_01762	12	cytC, cytochrome c
PPL_00779	10	cpiC, cystatin A3, putative cysteine protease inhibitor

Table A2: List of primers used in this study.

SACGB/GenBank accession number	Primers (5'→3')	Product
PPL_00912	GATACATCGATCCAAGAGATCCAGCTC CAACAACATACTATCAGAATTATTCGG	230 bp
PPL_03784	CCACTACACAATACCATTGAACCTAAC GAAATTTCAACCATCTTATTGTGTTGG	234 bp
PPL_05354	GTCAACACCACCACCGGACAATGTG GTTTGGACATTACATTGGTCGTAGGTG	236 bp
PPL_05833	CAAACCTCCACAAGAAGGCAGCTCGTC CTGTCCAAGTTGGTCTCTCTGAAAGCG	202 bp
PPL_09347	GGTGTATGGGCATGTAGTCCAATAAAC CAGTCTCCACCTTGTCTACCATAGATAC	176 bp
PPL_12248	GGCACCACAACCATTTTCTCAAGGAG CCTCTGAGGTGATTGAAATCAAATGC	192 bp
PPL_12271	CCAACCGCCGAAGACATAGAAGCAGTG CGCTCGACCGATGGTTCGTCGGCGGTG	242 bp
PPL_12370	CAAGTTCGACGTCTGGGGTGACACTG GAGTGGAAGATGTTCGTTGATCTCATTG	261 bp
PPL_01657, (*acaA2)*	CCTCGAGAATGCAGACCTCAGCGCCAC CCAGTGGTTTGGTAGTTGGTCTATTCC	261 bp
PPL_05049, *(pkaC)*	CTGCTGACAGAACACGTAGACTTGGTG CACATTGGTTCCTCATCGTATTTCTC	274 bp
PPL_10234, *(pdsA)*	CAACAATCTCATTAATATTACATTCCC CTTTTACCATACCTAACAGACCACCTTC	218 bp
EU797668	CTTGGTGTGTTGGATATTCGCGGTCATC GTTGCGCTCGAGACGCTTCGCTGTGTC	232 bp
PPL_04108	CAGTGCAGCACGTAGTCCAATCGCTG CATCGGAATCAAAGGTGGCGTGTATTG	197 bp
PPL_00855	GTAACAAATATACATCATGATAATAGG CATTCTTAAATAAGGTGGTCCTTTGAC	225 bp
PPL_02780	CACCAGCTAACCCAGCAGCTGGTATCTG CTTTACATTTATCTGCGTCTACTGGTGG	198 bp
PPL_02774	CCAGCAATCCTTGCCAAGGCGTAGAATG AGTATCACAACAACGGTGTTCATAGATG	236 bp
PPL_11763	CTACTCAATACACACCGATGAGACGTAC GTTGAGTGTTGGCAGATGAAGTAGTATC	249 bp
PPL_03541	GGATCAGTCTACAATGCTAATAGATCTC CCATTCCATCAGTTGTTCTCCTTGACC	216 bp
PPL_07908	GATGGGGATGTTGGTGGTGTCAAGTTG GAAGCAACAGCAGCAGTGATATCGAAC	172 bp
PPL_05702	CTGGAAGGGTGGTGTCGTTGATGGTTTG CGGTTGGTGGGAAAACACCTGGGATGG	242 bp
PPL_05195	GGATCGTGTAAAATCTGGAGTTTCATTG CTCCAACTCTAAGGTCCTTGAAACATCC	206 bp

PPL_12249	CAGCAATCCATTGTACATATGTAAGAAG GGATATTGTGTTTGGTGATTTAGTGG	188 bp
PPL_06644	ATACCTTGGCATGGCCATACCTAAAGGG GTGAACTTTGATGATACTTGAGTGCGT	190 bp
PPL_00902	CATAGATCTCGAAGAGTTTGAAGAGAC CTGTGGAAGACCGTCGTCTTCTACTGG	232 bp
PPL_05727	CCACCTACGACTCACAAATCATTATC CATTTCGACTGGACCCTTGGTTTGAC	197 bp
PPL_08454	CCATGTTACTTCTCTTTGTACCAAAG CATCACTATCAAATTGTGCATGGATAG	219 bp
PPL_08455	TTTTCATTCCAAAATTCCTTCGTATTGC GATTATTATCGGTTTGGTTGGACTTGG	225 bp
PPL_03564	GTATCGTAGAGATCCCAGAGTTTAAC CTCCAGAATTTTGGTACGAATAGAATG	199 bp
ITS_Pp	GAGGAAGGAGAAGTCGTAACAAGGTATC GCTTACTGATATGCTTAAGTTCAGCGGG	1000 bp

Table A3: Changes of gene expression in starving cells (1292 genes).

P. pallidum PN500 cells were starved for 2 or 3 hr without glorin. Listed are genes that were >3-fold regulated after 2 hr of starvation. The same list is compared to gene expression one hr later (note that many genes are below the >3-fold criterion after 3 hr). Note that 837 genes of 1292 (65%) genes are regulated >3-fold up or down after both 2 and 3 hours of starvation.

Gene ID	RPKM t0	RPKM t2	RPKM t3	fold change t2/t0	fold change t3/t0
PPL_02774	0.192	283.820	370.858	1476.512	1929.304
PPL_02780	0.248	276.561	377.055	1114.751	1519.820
PPL_07908	0.266	163.753	133.324	615.725	501.309
PPL_09544	0.992	378.066	318.751	381.124	321.329
PPL_00254	1.122	399.002	284.051	355.517	253.094
PPL_00132	0.117	26.541	7.313	226.387	62.378
PPL_04306	6.108	1281.763	1049.866	209.856	171.889
PPL_03342	0.232	47.961	17.526	206.993	75.640
PPL_04307	13.385	1636.434	1296.892	122.256	96.889
PPL_02038	3.208	384.521	295.730	119.856	92.179
PPL_06550	1.178	107.991	106.252	91.667	90.191
PPL_04586	0.714	41.435	54.630	58.055	76.544
PPL_03576	0.074	4.289	2.647	57.953	35.767
PPL_02271	1.564	87.558	84.027	55.979	53.722
PPL_04882	0.768	42.031	27.333	54.751	35.605
PPL_10681	1.121	60.605	16.830	54.056	15.011
PPL_06523	0.139	7.506	3.256	53.823	23.348
PPL_02260	4.023	205.156	242.138	50.993	60.186
PPL_09188	1.292	65.583	24.588	50.771	19.034
PPL_06520	0.319	15.438	7.421	48.434	23.283
PPL_06519	0.367	17.686	13.363	48.168	36.395
PPL_04835	1.754	81.059	9.893	46.204	5.639
PPL_04774	0.070	3.131	1.921	44.838	27.517
PPL_09143	2.707	117.785	102.047	43.516	37.702
PPL_10531	0.021	0.874	0.577	41.972	27.724
PPL_03183	0.570	23.047	26.627	40.465	46.751
PPL_01039	22.087	832.893	656.853	37.709	29.739
PPL_09787	9.891	365.735	172.666	36.977	17.457
PPL_09781	0.275	9.622	2.429	35.027	8.843
PPL_03343	0.179	5.824	3.365	32.467	18.759
PPL_03929	0.262	8.315	4.518	31.733	17.243
PPL_03184	0.427	12.359	12.403	28.968	29.070
PPL_02069	27.146	781.723	418.791	28.797	15.427
PPL_08488	0.961	26.933	15.832	28.040	16.482
PPL_03638	18.118	499.623	764.683	27.577	42.206
PPL_01716	0.063	1.672	1.415	26.565	22.472
PPL_05982	0.950	24.665	6.570	25.969	6.917
PPL_02528	0.270	6.908	3.786	25.596	14.029
PPL_11771	2.542	64.853	75.165	25.513	29.570

PPL_01209	0.218	5.506	3.153	25.293	14.483
PPL_02367	0.248	6.271	5.327	25.259	21.456
PPL_10757	1.354	33.574	26.288	24.791	19.411
PPL_08807	4.890	118.135	52.563	24.156	10.748
PPL_00578	0.692	16.604	11.319	24.001	16.361
PPL_09690	0.568	13.574	3.825	23.908	6.736
PPL_00953	0.605	13.556	21.897	22.405	36.192
PPL_02536	10.753	239.839	302.725	22.305	28.153
PPL_02150	1.066	23.485	7.819	22.039	7.338
PPL_11512	6.502	142.697	53.915	21.946	8.292
PPL_02247	0.558	12.169	7.675	21.815	13.758
PPL_04010	0.079	1.721	1.880	21.772	23.792
PPL_06900	4.443	94.314	67.822	21.230	15.267
PPL_09458	0.257	5.346	1.292	20.767	5.019
PPL_00731	1.158	23.848	5.827	20.599	5.033
PPL_00716	0.209	4.128	1.089	19.739	5.207
PPL_11666	0.401	7.897	10.651	19.671	26.529
PPL_09745	0.023	0.441	0.460	19.183	20.008
PPL_06552	2.583	49.525	74.177	19.170	28.712
PPL_10845	0.092	1.730	0.907	18.799	9.860
PPL_00538	71.055	1332.896	1376.547	18.759	19.373
PPL_00051	0.093	1.742	1.604	18.684	17.200
PPL_12476	1.800	32.930	20.032	18.290	11.126
PPL_05902	0.672	12.190	47.110	18.149	70.139
PPL_00519	0.106	1.916	2.116	18.111	20.007
PPL_10560	0.814	14.563	8.957	17.892	11.005
PPL_02982	1.639	29.142	16.994	17.781	10.368
PPL_02248	0.236	4.051	2.410	17.132	10.194
PPL_05194	0.164	2.799	0.722	17.092	4.411
PPL_10461	0.585	9.982	4.033	17.069	6.895
PPL_02657	0.664	11.100	5.052	16.721	7.610
PPL_09440	1.229	20.547	10.163	16.717	8.268
PPL_00372	0.115	1.908	1.014	16.662	8.854
PPL_00715	1.607	26.613	4.404	16.558	2.740
PPL_02781	1.125	18.197	15.720	16.179	13.976
PPL_04947	0.067	1.066	0.836	15.955	12.506
PPL_03840	0.665	10.611	7.605	15.950	11.433
PPL_04785	2.926	46.546	40.309	15.909	13.777
PPL_08268	0.924	14.542	14.015	15.743	15.173
PPL_08352	0.447	7.000	2.455	15.656	5.491
PPL_08805	3.236	50.069	65.538	15.474	20.255
PPL_04412	0.571	8.806	5.075	15.420	8.888
PPL_08689	12.015	183.535	57.631	15.275	4.797
PPL_04356	30.734	461.288	151.690	15.009	4.935
PPL_04193	0.914	13.682	17.098	14.972	18.710
PPL_07733	16.493	244.244	103.609	14.809	6.282
PPL_03785	0.031	0.440	0.527	14.225	17.009
PPL_11193	38.447	545.655	569.936	14.193	14.824
PPL_02633	45.259	629.361	521.113	13.906	11.514
PPL_09814	28.004	384.807	302.224	13.741	10.792
PPL_08361	4.737	64.947	42.283	13.711	8.926
PPL_06494	0.093	1.268	0.353	13.599	3.782

PPL_10714	2.830	38.377	27.991	13.560	9.890
PPL_00292	1.447	19.228	16.804	13.286	11.611
PPL_07200	0.071	0.938	0.391	13.193	5.501
PPL_10561	0.239	3.136	2.632	13.113	11.003
PPL_11798	0.471	6.120	4.932	13.001	10.477
PPL_05600	1.941	25.206	9.988	12.988	5.147
PPL_07885	0.193	2.508	3.828	12.979	19.815
PPL_02784	0.685	8.667	9.049	12.661	13.218
PPL_11792	1.977	24.917	19.288	12.602	9.755
PPL_05680	0.236	2.934	2.182	12.421	9.235
PPL_05195	5.658	69.666	117.677	12.313	20.798
PPL_04271	0.925	11.215	9.825	12.129	10.626
PPL_12532	0.850	10.303	8.909	12.117	10.477
PPL_07910	0.049	0.588	0.681	12.081	13.995
PPL_00405	5.411	64.608	17.936	11.939	3.314
PPL_04524	125.867	1498.588	2068.794	11.906	16.436
PPL_10555	0.171	2.039	1.710	11.896	9.975
PPL_09702	3.691	43.732	19.584	11.847	5.306
PPL_05932	20.783	245.908	106.781	11.832	5.138
PPL_02039	11.818	138.307	130.314	11.703	11.026
PPL_08429	6.523	76.312	30.830	11.698	4.726
PPL_08089	0.047	0.553	0.238	11.687	5.041
PPL_09134	0.564	6.579	4.926	11.669	8.737
PPL_11490	5.753	66.634	123.843	11.584	21.528
PPL_03571	13.594	151.313	33.721	11.131	2.481
PPL_07634	0.037	0.415	0.131	11.089	3.501
PPL_05961	1.223	13.366	10.703	10.930	8.752
PPL_12019	4.439	48.081	36.440	10.832	8.209
PPL_00052	6.104	65.793	72.431	10.779	11.866
PPL_01229	0.100	1.069	1.455	10.679	14.540
PPL_10932	0.090	0.964	0.488	10.653	5.390
PPL_05299	5.634	59.725	42.868	10.601	7.609
PPL_05174	9.682	102.295	79.247	10.565	8.185
PPL_02541	0.996	10.472	12.271	10.509	12.315
PPL_06374	0.427	4.462	1.642	10.440	3.841
PPL_02535	0.112	1.162	0.790	10.416	7.080
PPL_00367	0.574	5.946	6.097	10.351	10.613
PPL_08647	1.899	19.482	13.229	10.259	6.967
PPL_05601	0.415	4.232	2.143	10.186	5.159
PPL_03341	0.361	3.669	1.788	10.171	4.955
PPL_09762	2.965	30.096	31.752	10.151	10.710
PPL_04438	1.363	13.776	12.393	10.104	9.090
PPL_09662	0.130	1.310	0.954	10.037	7.309
PPL_01872	0.175	1.728	1.090	9.865	6.224
PPL_10462	0.285	2.793	2.134	9.815	7.499
PPL_07129	0.195	1.890	0.943	9.701	4.838
PPL_05405	4.540	43.865	55.887	9.661	12.309
PPL_12333	17.073	164.932	118.068	9.660	6.915
PPL_02846	330.946	3173.149	2483.372	9.588	7.504
PPL_08265	7.685	73.083	30.960	9.510	4.029
PPL_11833	2.260	21.361	19.688	9.451	8.711
PPL_01305	4.025	37.514	48.298	9.319	11.998

PPL_11559	1.196	11.108	14.145	9.288	11.827
PPL_10680	0.824	7.574	2.555	9.196	3.103
PPL_12442	1.723	15.811	13.618	9.175	7.903
PPL_06890	0.090	0.824	1.167	9.126	12.919
PPL_02234	0.469	4.267	3.001	9.102	6.402
PPL_07203	10.070	90.752	126.865	9.012	12.598
PPL_06178	0.226	2.033	0.921	9.012	4.085
PPL_09437	0.207	1.866	1.462	9.004	7.055
PPL_06442	0.367	3.295	0.917	8.984	2.501
PPL_04766	0.119	1.070	0.681	8.975	5.717
PPL_11065	0.686	6.119	2.167	8.922	3.159
PPL_10945	13.243	116.379	106.186	8.788	8.018
PPL_05486	0.526	4.609	4.564	8.763	8.678
PPL_01952	0.233	2.038	1.410	8.737	6.046
PPL_07597	2.126	18.549	20.756	8.725	9.763
PPL_07057	6.080	52.754	33.352	8.677	5.486
PPL_09894	0.209	1.809	0.912	8.670	4.371
PPL_03107	0.148	1.274	0.679	8.633	4.603
PPL_12370	0.259	2.229	1.335	8.612	5.156
PPL_01146	8.346	71.607	69.605	8.579	8.340
PPL_02281	8.208	69.942	43.838	8.521	5.341
PPL_03752	0.240	2.044	1.803	8.513	7.510
PPL_11990	1.141	9.646	9.553	8.455	8.373
PPL_02628	5.275	44.349	11.568	8.408	2.193
PPL_00855	0.206	1.727	2.661	8.399	12.939
PPL_01117	0.843	7.078	5.524	8.392	6.550
PPL_08056	234.038	1949.188	2075.470	8.329	8.868
PPL_09695	55.525	461.751	555.589	8.316	10.006
PPL_09054	1.247	10.366	6.164	8.310	4.941
PPL_02950	0.956	7.883	5.335	8.243	5.578
PPL_05337	4.206	34.661	36.681	8.240	8.721
PPL_12554	0.353	2.898	1.042	8.219	2.955
PPL_05917	0.159	1.297	0.545	8.165	3.430
PPL_12216	0.129	1.051	0.516	8.155	4.000
PPL_09724	4.279	34.790	14.570	8.131	3.405
PPL_12381	98.103	796.042	607.292	8.114	6.190
PPL_00057	7.229	58.529	81.436	8.096	11.265
PPL_05394	0.049	0.399	0.394	8.094	8.003
PPL_00085	0.055	0.445	0.358	8.094	6.502
PPL_09260	884.568	7150.300	7073.941	8.083	7.997
PPL_01361	0.122	0.984	0.603	8.064	4.942
PPL_03340	0.279	2.239	1.845	8.014	6.603
PPL_00396	0.767	6.094	5.643	7.949	7.362
PPL_10074	16.208	128.557	106.539	7.931	6.573
PPL_12030	0.039	0.307	0.272	7.892	7.000
PPL_04158	0.141	1.112	1.771	7.890	12.566
PPL_00368	1.949	15.377	14.102	7.889	7.235
PPL_04042	4.778	37.560	18.452	7.862	3.862
PPL_07388	0.196	1.542	1.175	7.858	5.986
PPL_10626	36.444	285.977	95.263	7.847	2.614
PPL_09786	0.257	2.013	1.265	7.837	4.924
PPL_01365	0.942	7.376	4.748	7.830	5.041

PPL_12169	0.133	1.039	0.783	7.813	5.890
PPL_06502	0.660	5.126	3.279	7.764	4.966
PPL_10928	0.180	1.396	0.888	7.749	4.930
PPL_09010	0.446	3.446	2.016	7.722	4.518
PPL_00514	7.253	55.967	93.525	7.716	12.894
PPL_06920	0.815	6.245	3.811	7.666	4.678
PPL_10158	0.228	1.749	1.578	7.663	6.910
PPL_03356	0.043	0.325	0.235	7.628	5.501
PPL_11295	0.084	0.639	0.545	7.628	6.504
PPL_10595	0.099	0.756	0.460	7.612	4.631
PPL_05113	0.251	1.908	1.259	7.606	5.019
PPL_03339	19.950	151.101	34.276	7.574	1.718
PPL_06996	279.376	2114.808	895.883	7.570	3.207
PPL_12525	2.107	15.909	18.790	7.550	8.917
PPL_00378	0.103	0.770	0.302	7.476	2.935
PPL_06684	17.116	127.829	105.543	7.469	6.166
PPL_05364	0.950	7.053	1.266	7.427	1.334
PPL_10866	0.070	0.513	0.496	7.367	7.131
PPL_11865	0.179	1.313	3.297	7.332	18.418
PPL_11523	1.314	9.633	4.671	7.329	3.554
PPL_08603	0.642	4.663	1.948	7.263	3.034
PPL_04124	0.171	1.240	1.457	7.235	8.499
PPL_10805	0.239	1.728	1.077	7.224	4.502
PPL_02839	0.112	0.811	0.590	7.221	5.251
PPL_12463	11.569	83.530	61.498	7.220	5.316
PPL_02903	13.117	94.534	82.971	7.207	6.325
PPL_08043	9.078	65.116	44.484	7.173	4.900
PPL_07198	0.256	1.828	0.577	7.152	2.260
PPL_06066	0.535	3.811	2.697	7.129	5.044
PPL_03605	0.224	1.596	0.758	7.128	3.387
PPL_05105	1.351	9.583	6.716	7.092	4.970
PPL_08395	53.105	375.800	319.215	7.077	6.011
PPL_01720	0.688	4.862	3.273	7.062	4.754
PPL_02300	7.782	54.832	57.910	7.046	7.442
PPL_11965	0.178	1.248	1.512	7.029	8.513
PPL_09575	0.233	1.632	2.327	6.998	9.977
PPL_01233	0.137	0.953	0.519	6.946	3.781
PPL_08054	1.189	8.258	5.379	6.946	4.524
PPL_06383	34.472	239.265	86.907	6.941	2.521
PPL_06907	2.068	14.350	5.793	6.938	2.801
PPL_11493	0.634	4.397	3.620	6.932	5.707
PPL_09869	0.355	2.444	1.621	6.892	4.570
PPL_00758	0.258	1.777	1.612	6.890	6.252
PPL_05298	6.908	47.586	39.069	6.889	5.656
PPL_05755	2.298	15.819	15.654	6.884	6.812
PPL_04321	0.619	4.245	6.315	6.859	10.204
PPL_08350	1.358	9.285	6.654	6.840	4.901
PPL_00136	8.855	60.501	72.238	6.832	8.158
PPL_02325	0.659	4.501	2.523	6.830	3.828
PPL_09364	3.135	21.376	17.554	6.818	5.599
PPL_06434	0.326	2.212	1.552	6.795	4.768
PPL_08476	0.967	6.559	9.544	6.781	9.867

PPL_03841	1.787	12.079	7.464	6.760	4.177
PPL_06230	0.294	1.984	0.603	6.752	2.052
PPL_08480	1.456	9.739	2.553	6.689	1.753
PPL_08317	1.438	9.610	10.084	6.681	7.011
PPL_02939	0.327	2.178	1.169	6.666	3.578
PPL_05833	0.517	3.441	7.650	6.661	14.808
PPL_11240	0.545	3.629	3.823	6.659	7.014
PPL_11992	1.867	12.425	7.119	6.654	3.813
PPL_05855	15.774	104.966	35.133	6.654	2.227
PPL_05877	1.547	10.275	9.417	6.643	6.088
PPL_07208	0.249	1.653	0.809	6.632	3.247
PPL_08967	8.548	56.651	58.923	6.627	6.893
PPL_00746	12.970	85.610	91.786	6.600	7.077
PPL_07879	6.250	41.191	38.970	6.590	6.235
PPL_07232	1.411	9.274	11.406	6.573	8.085
PPL_06848	1.855	12.158	10.753	6.554	5.797
PPL_05333	2.358	15.452	32.013	6.552	13.574
PPL_09816	10.305	67.316	45.287	6.533	4.395
PPL_02211	0.715	4.661	3.949	6.515	5.520
PPL_04148	1.758	11.440	8.572	6.507	4.875
PPL_02377	2.949	19.184	15.533	6.506	5.267
PPL_08134	0.766	4.947	3.183	6.457	4.155
PPL_12462	1.235	7.972	6.892	6.453	5.579
PPL_10943	0.673	4.331	4.259	6.434	6.327
PPL_08309	22.049	141.724	100.292	6.428	4.549
PPL_07920	2.309	14.779	10.839	6.401	4.694
PPL_09924	0.270	1.729	1.399	6.399	5.177
PPL_08973	0.441	2.821	1.856	6.396	4.209
PPL_06732	0.307	1.960	2.173	6.390	7.084
PPL_01566	0.292	1.864	1.111	6.379	3.802
PPL_11328	0.802	5.106	3.200	6.366	3.990
PPL_04125	1.441	9.136	1.718	6.342	1.193
PPL_04413	0.252	1.584	1.192	6.294	4.736
PPL_07939	3.283	20.644	15.990	6.288	4.871
PPL_02856	39.361	247.447	140.397	6.287	3.567
PPL_00897	0.284	1.786	1.495	6.281	5.258
PPL_09743	0.927	5.816	4.445	6.277	4.797
PPL_02658	1.871	11.692	7.821	6.249	4.181
PPL_07084	20.861	130.291	63.179	6.246	3.029
PPL_07014	0.110	0.688	0.752	6.242	6.820
PPL_08856	1.181	7.364	7.291	6.238	6.176
PPL_07365	2.972	18.372	15.102	6.182	5.082
PPL_03471	0.488	3.014	2.263	6.180	4.640
PPL_07687	0.125	0.774	0.460	6.172	3.667
PPL_09878	1.641	10.114	7.001	6.163	4.266
PPL_00612	22.149	136.175	90.452	6.148	4.084
PPL_03291	0.254	1.562	0.668	6.139	2.625
PPL_10426	0.059	0.363	0.366	6.118	6.170
PPL_08861	28.815	176.050	118.088	6.110	4.098
PPL_02872	0.281	1.710	1.327	6.092	4.726
PPL_09865	0.035	0.216	0.213	6.091	6.001
PPL_08648	1.717	10.447	7.612	6.086	4.434

PPL_05017	0.317	1.926	0.869	6.077	2.741
PPL_09639	0.724	4.382	2.199	6.056	3.040
PPL_04565	4.225	25.543	30.510	6.046	7.222
PPL_08615	0.547	3.309	1.601	6.045	2.924
PPL_03292	49.089	296.287	133.761	6.036	2.725
PPL_08534	19.085	115.028	109.427	6.027	5.734
PPL_11597	93.622	563.686	258.143	6.021	2.757
PPL_00798	1.355	8.151	3.551	6.017	2.621
PPL_06696	0.261	1.565	1.255	5.993	4.805
PPL_00125	0.540	3.237	1.922	5.993	3.558
PPL_02062	0.339	2.032	1.882	5.990	5.548
PPL_06029	0.650	3.888	3.918	5.980	6.025
PPL_03338	2.686	16.033	5.367	5.970	1.998
PPL_04883	0.057	0.341	0.041	5.960	0.708
PPL_01778	1.855	11.042	11.679	5.953	6.296
PPL_10576	26.872	159.803	212.746	5.947	7.917
PPL_06627	0.031	0.182	0.184	5.929	6.004
PPL_10847	0.176	1.041	1.142	5.929	6.503
PPL_08369	19.898	117.542	75.084	5.907	3.773
PPL_10608	0.802	4.734	3.966	5.906	4.948
PPL_02583	11.528	67.815	29.029	5.883	2.518
PPL_12443	2.657	15.613	9.611	5.877	3.618
PPL_02037	0.541	3.178	1.941	5.871	3.586
PPL_11619	51.888	304.579	216.638	5.870	4.175
PPL_07775	3.112	18.261	16.341	5.868	5.251
PPL_05311	3.456	20.233	16.766	5.854	4.851
PPL_12310	32.442	189.313	296.441	5.835	9.138
PPL_01693	0.387	2.252	1.612	5.823	4.168
PPL_10539	2.792	16.256	11.509	5.822	4.122
PPL_04390	1.417	8.251	4.860	5.821	3.429
PPL_02951	1.483	8.625	6.238	5.816	4.207
PPL_05679	1.153	6.701	5.021	5.812	4.355
PPL_10894	0.319	1.851	1.356	5.800	4.251
PPL_06106	5.038	29.174	28.070	5.791	5.572
PPL_12011	0.161	0.929	0.455	5.781	2.834
PPL_04588	3.543	20.471	10.460	5.778	2.953
PPL_10520	0.738	4.245	3.889	5.752	5.269
PPL_11400	5.221	30.004	58.195	5.747	11.147
PPL_09106	1.006	5.774	5.454	5.740	5.422
PPL_00648	21.558	123.706	107.751	5.738	4.998
PPL_07447	0.204	1.167	0.407	5.727	2.000
PPL_10979	0.220	1.260	0.660	5.727	2.999
PPL_11349	0.043	0.248	0.424	5.719	9.760
PPL_05265	0.204	1.155	0.764	5.671	3.748
PPL_09438	5.175	29.242	19.087	5.651	3.688
PPL_11135	23.500	132.614	35.603	5.643	1.515
PPL_06896	7.601	42.865	33.202	5.639	4.368
PPL_12151	4.636	26.133	13.454	5.636	2.902
PPL_11401	3.915	22.049	32.889	5.632	8.401
PPL_00916	0.418	2.348	1.987	5.618	4.754
PPL_03314	59.867	335.345	363.527	5.602	6.072
PPL_08940	172.592	966.814	579.757	5.602	3.359

PPL_09436	13.699	76.439	88.597	5.580	6.467
PPL_01401	8.749	48.606	38.499	5.555	4.400
PPL_12175	0.276	1.533	0.516	5.545	1.865
PPL_10947	2.153	11.914	8.512	5.533	3.953
PPL_03860	51.752	286.178	296.263	5.530	5.725
PPL_04644	61.076	337.251	478.432	5.522	7.833
PPL_04006	0.444	2.449	2.358	5.516	5.312
PPL_06319	0.101	0.559	0.998	5.511	9.841
PPL_05987	0.584	3.209	2.308	5.496	3.953
PPL_02824	1.380	7.578	7.238	5.493	5.246
PPL_01133	0.696	3.822	2.780	5.488	3.992
PPL_04942	1.248	6.830	6.420	5.474	5.146
PPL_07240	15.983	87.297	90.310	5.462	5.650
PPL_09638	2.531	13.818	7.780	5.460	3.074
PPL_06018	1.410	7.681	6.878	5.447	4.878
PPL_04260	0.900	4.885	4.189	5.428	4.655
PPL_00087	0.668	3.617	2.639	5.417	3.953
PPL_06292	1.136	6.148	5.869	5.411	5.165
PPL_01786	13.140	71.061	53.788	5.408	4.093
PPL_07745	1.990	10.705	9.375	5.378	4.710
PPL_00053	6.075	32.581	46.736	5.363	7.694
PPL_02720	0.181	0.972	1.088	5.362	6.001
PPL_01419	0.569	3.051	3.416	5.362	6.003
PPL_03943	1.933	10.351	8.207	5.355	4.246
PPL_05153	12.187	65.094	48.529	5.341	3.982
PPL_09630	1.369	7.302	6.689	5.334	4.887
PPL_00137	0.162	0.862	0.917	5.328	5.670
PPL_10964	0.287	1.528	1.080	5.327	3.766
PPL_08494	4.871	25.927	26.136	5.322	5.365
PPL_06598	0.331	1.759	1.405	5.317	4.248
PPL_02138	1.615	8.587	8.274	5.316	5.122
PPL_02157	0.287	1.522	1.621	5.307	5.649
PPL_05651	1.015	5.369	3.698	5.290	3.644
PPL_00029	3.411	18.034	16.908	5.288	4.957
PPL_10059	3.599	19.018	4.442	5.285	1.234
PPL_04465	1.321	6.978	10.235	5.284	7.750
PPL_06134	0.498	2.632	2.085	5.279	4.182
PPL_10890	3.134	16.506	15.791	5.266	5.038
PPL_10516	0.069	0.362	0.241	5.261	3.501
PPL_03721	0.220	1.158	0.770	5.261	3.500
PPL_00545	2.334	12.266	6.622	5.256	2.837
PPL_11245	1.025	5.380	4.173	5.251	4.073
PPL_05469	5.755	30.140	12.514	5.237	2.175
PPL_03307	0.585	3.063	1.739	5.236	2.973
PPL_05305	0.201	1.050	0.720	5.234	3.593
PPL_09780	5.353	28.009	11.363	5.233	2.123
PPL_01943	2.653	13.850	11.059	5.220	4.168
PPL_09220	125.010	652.143	459.886	5.217	3.679
PPL_09658	0.278	1.448	1.057	5.207	3.802
PPL_12204	0.981	5.103	3.063	5.205	3.123
PPL_12089	3.261	16.970	6.420	5.204	1.969
PPL_03561	3.759	19.557	18.635	5.202	4.957

PPL_02558	156.082	811.930	618.048	5.202	3.960
PPL_08058	0.079	0.413	0.163	5.200	2.048
PPL_04807	0.818	4.252	3.661	5.198	4.476
PPL_07011	0.530	2.744	1.932	5.179	3.647
PPL_08784	6.059	31.365	24.854	5.176	4.102
PPL_04534	1.205	6.234	8.013	5.173	6.650
PPL_02876	4.575	23.619	20.971	5.163	4.584
PPL_10787	0.065	0.338	0.262	5.160	4.000
PPL_10224	45.657	235.423	99.682	5.156	2.183
PPL_06229	14.870	76.646	36.911	5.154	2.482
PPL_04874	0.156	0.805	0.723	5.152	4.629
PPL_09720	16.851	86.699	50.295	5.145	2.985
PPL_10573	0.228	1.169	1.077	5.130	4.725
PPL_06757	2.076	10.643	8.876	5.126	4.275
PPL_09185	9.731	49.779	37.118	5.115	3.814
PPL_10314	13.721	69.902	28.431	5.095	2.072
PPL_09719	9.205	46.861	27.383	5.091	2.975
PPL_12368	13.994	71.220	52.134	5.089	3.725
PPL_09817	12.237	62.264	37.561	5.088	3.069
PPL_02685	9.522	48.375	39.918	5.080	4.192
PPL_12497	1.458	7.402	4.548	5.076	3.119
PPL_06823	0.155	0.787	0.505	5.076	3.259
PPL_01948	1.121	5.678	4.510	5.066	4.024
PPL_03088	1.325	6.715	4.858	5.066	3.666
PPL_11510	6.133	31.066	36.246	5.065	5.910
PPL_06044	6.263	31.669	51.009	5.057	8.145
PPL_00666	0.203	1.022	0.574	5.043	2.833
PPL_00700	0.135	0.679	0.525	5.043	3.895
PPL_11334	15.386	77.490	76.715	5.036	4.986
PPL_05323	0.414	2.084	1.694	5.029	4.088
PPL_09319	6.079	30.562	27.574	5.028	4.536
PPL_01699	32.299	161.921	112.480	5.013	3.482
PPL_03046	1.068	5.332	5.458	4.992	5.109
PPL_09653	4.910	24.481	20.921	4.986	4.261
PPL_12279	0.325	1.620	1.032	4.985	3.174
PPL_01733	0.561	2.788	2.037	4.969	3.631
PPL_02229	1.057	5.249	4.478	4.968	4.238
PPL_10519	2.401	11.874	3.764	4.945	1.567
PPL_07239	3.755	18.562	20.736	4.943	5.522
PPL_10271	7.017	34.646	32.299	4.937	4.603
PPL_10588	114.068	562.760	271.753	4.934	2.382
PPL_01755	0.752	3.710	2.435	4.934	3.238
PPL_02456	0.858	4.228	3.312	4.928	3.861
PPL_11746	1.182	5.818	5.496	4.921	4.649
PPL_01532	2.112	10.341	9.061	4.896	4.290
PPL_05115	3.152	15.372	10.696	4.878	3.394
PPL_09751	0.644	3.138	1.666	4.876	2.589
PPL_05632	2.160	10.531	7.474	4.876	3.461
PPL_08023	1.090	5.312	3.918	4.872	3.593
PPL_11623	0.275	1.340	0.746	4.864	2.706
PPL_01915	19.655	95.515	46.180	4.860	2.350
PPL_07250	0.650	3.160	1.896	4.859	2.915

PPL_06369	3.952	19.160	12.466	4.848	3.154
PPL_09863	2.351	11.392	27.442	4.845	11.672
PPL_07238	1.655	8.007	7.004	4.838	4.232
PPL_07880	0.120	0.583	0.249	4.837	2.069
PPL_11094	0.100	0.482	0.191	4.835	1.918
PPL_10270	18.174	87.815	74.467	4.832	4.098
PPL_03912	1.636	7.890	6.019	4.823	3.679
PPL_04129	0.472	2.273	1.548	4.819	3.283
PPL_03025	11.770	56.577	51.093	4.807	4.341
PPL_05842	5.015	24.086	20.518	4.803	4.091
PPL_04971	1.201	5.764	5.371	4.801	4.473
PPL_03618	1.913	9.181	3.511	4.800	1.835
PPL_09426	0.083	0.400	0.625	4.796	7.505
PPL_03441	1.278	6.103	5.943	4.777	4.652
PPL_09624	0.716	3.419	2.819	4.776	3.938
PPL_07912	0.244	1.163	0.960	4.773	3.939
PPL_07541	0.433	2.055	1.805	4.744	4.166
PPL_09487	62.917	298.306	343.343	4.741	5.457
PPL_01134	1.809	8.575	7.419	4.739	4.100
PPL_07165	8.194	38.758	47.798	4.730	5.834
PPL_00056	6.561	30.940	27.562	4.716	4.201
PPL_08594	0.280	1.323	0.838	4.716	2.990
PPL_11997	2.248	10.595	9.187	4.713	4.087
PPL_00403	5.034	23.687	21.716	4.706	4.314
PPL_03582	3.386	15.911	18.236	4.700	5.386
PPL_11862	0.553	2.598	6.055	4.696	10.943
PPL_00878	0.958	4.500	2.381	4.696	2.485
PPL_10846	0.051	0.240	0.256	4.695	5.002
PPL_06403	4.100	19.229	29.141	4.690	7.108
PPL_07697	0.807	3.782	2.781	4.687	3.446
PPL_03325	1.659	7.774	5.552	4.685	3.346
PPL_11042	0.398	1.864	1.417	4.681	3.558
PPL_02615	0.113	0.527	0.311	4.670	2.759
PPL_05849	0.453	2.112	1.168	4.659	2.576
PPL_06382	1.159	5.398	3.755	4.658	3.240
PPL_02460	1.805	8.401	8.792	4.655	4.872
PPL_03826	1.053	4.894	3.232	4.649	3.069
PPL_10809	0.116	0.538	0.410	4.642	3.540
PPL_01002	1.322	6.121	5.964	4.629	4.510
PPL_07045	1.707	7.890	7.624	4.622	4.466
PPL_10259	0.517	2.382	1.475	4.611	2.855
PPL_01703	0.241	1.110	0.720	4.609	2.989
PPL_11984	0.508	2.342	1.375	4.606	2.705
PPL_11893	2.932	13.498	11.844	4.605	4.040
PPL_00175	1.285	5.918	4.851	4.605	3.775
PPL_09251	1.037	4.758	3.937	4.589	3.797
PPL_08051	1.658	7.598	8.374	4.582	5.050
PPL_11239	3.168	14.488	12.751	4.574	4.026
PPL_03316	141.314	646.147	697.645	4.572	4.937
PPL_10637	7.131	32.605	41.265	4.572	5.786
PPL_01058	0.138	0.631	0.413	4.570	2.988
PPL_01295	2.165	9.894	8.230	4.570	3.802

PPL_00373	1.493	6.816	4.990	4.564	3.342
PPL_04630	7.901	36.037	36.240	4.561	4.587
PPL_00383	0.296	1.351	1.439	4.561	4.858
PPL_11671	0.172	0.784	0.401	4.560	2.333
PPL_04532	1.564	7.116	3.724	4.549	2.381
PPL_09289	0.824	3.746	2.389	4.546	2.900
PPL_06701	1.222	5.524	3.175	4.521	2.598
PPL_05180	19.950	89.928	54.053	4.508	2.709
PPL_10361	2.059	9.254	8.856	4.495	4.301
PPL_06889	0.158	0.706	0.366	4.473	2.319
PPL_04993	0.969	4.329	6.129	4.467	6.324
PPL_05073	2.937	13.112	10.764	4.464	3.665
PPL_03617	8.618	38.465	14.580	4.463	1.692
PPL_05504	6.054	27.013	20.172	4.462	3.332
PPL_09798	0.169	0.756	0.537	4.459	3.170
PPL_11700	7.182	31.993	15.952	4.455	2.221
PPL_06982	6.002	26.722	19.734	4.452	3.288
PPL_06790	12.817	56.914	56.179	4.440	4.383
PPL_01263	2.894	12.798	10.303	4.423	3.561
PPL_07868	22.214	97.833	76.653	4.404	3.451
PPL_08018	7.613	33.522	35.597	4.403	4.676
PPL_02033	5.178	22.792	7.704	4.401	1.488
PPL_11972	0.404	1.777	1.474	4.397	3.645
PPL_11273	7.903	34.741	43.643	4.396	5.523
PPL_09904	2.015	8.853	5.838	4.394	2.897
PPL_12309	9.751	42.845	35.697	4.394	3.661
PPL_04374	0.053	0.234	0.188	4.392	3.533
PPL_04464	0.210	0.923	0.914	4.391	4.348
PPL_12621	0.228	0.998	0.720	4.383	3.161
PPL_11324	0.359	1.571	0.874	4.380	2.437
PPL_07124	72.274	316.065	86.672	4.373	1.199
PPL_07479	6.130	26.782	27.195	4.369	4.436
PPL_06125	2.703	11.781	8.381	4.358	3.100
PPL_01337	12.909	56.208	41.369	4.354	3.205
PPL_07157	15.520	67.452	46.283	4.346	2.982
PPL_05618	0.288	1.250	0.960	4.342	3.335
PPL_08699	8.049	34.907	25.605	4.337	3.181
PPL_09200	4.853	21.025	19.674	4.332	4.054
PPL_09815	2.307	9.960	7.533	4.318	3.266
PPL_10688	1.026	4.430	3.612	4.317	3.520
PPL_00856	0.165	0.711	1.704	4.316	10.346
PPL_11974	0.464	1.991	1.590	4.292	3.427
PPL_12065	0.349	1.496	1.046	4.290	3.001
PPL_01343	0.071	0.306	0.230	4.288	3.219
PPL_03778	6.834	29.263	23.889	4.282	3.495
PPL_09261	0.346	1.480	1.089	4.274	3.145
PPL_12472	3.463	14.795	12.030	4.272	3.474
PPL_08313	3.936	16.810	13.414	4.271	3.408
PPL_07478	9.573	40.874	67.774	4.270	7.080
PPL_04912	25.357	108.108	109.686	4.264	4.326
PPL_06010	14.746	62.723	47.017	4.254	3.188
PPL_10463	0.358	1.523	0.636	4.253	1.776

PPL_10747	0.830	3.529	3.269	4.252	3.938
PPL_01493	1.607	6.833	5.466	4.251	3.401
PPL_10267	3.537	15.014	21.533	4.245	6.088
PPL_07009	2.601	11.042	6.658	4.245	2.560
PPL_02444	2.604	11.049	16.167	4.244	6.210
PPL_01540	10.816	45.905	8.712	4.244	0.806
PPL_00090	0.220	0.933	0.763	4.238	3.466
PPL_02316	13.347	56.508	23.270	4.234	1.743
PPL_04742	0.232	0.983	0.349	4.229	1.500
PPL_02948	1.180	4.989	4.304	4.227	3.647
PPL_05771	1.587	6.704	5.567	4.225	3.508
PPL_09222	9.921	41.900	38.199	4.223	3.850
PPL_03766	0.993	4.190	3.499	4.221	3.524
PPL_09327	0.522	2.203	1.811	4.220	3.469
PPL_09067	0.582	2.446	1.866	4.205	3.208
PPL_05159	2.697	11.317	7.840	4.197	2.907
PPL_11946	0.515	2.160	2.016	4.195	3.916
PPL_04704	4.991	20.932	13.047	4.194	2.614
PPL_11906	17.921	75.061	65.427	4.189	3.651
PPL_11069	1.683	7.040	5.739	4.183	3.410
PPL_08477	7.825	32.677	50.321	4.176	6.431
PPL_02106	5.624	23.483	12.074	4.176	2.147
PPL_12471	14.636	61.066	45.197	4.172	3.088
PPL_05462	142.513	594.355	679.916	4.171	4.771
PPL_10893	1.897	7.911	6.301	4.169	3.321
PPL_06760	0.778	3.243	3.978	4.169	5.115
PPL_09774	0.098	0.408	0.429	4.168	4.381
PPL_01657	0.093	0.388	0.220	4.159	2.354
PPL_02105	148.051	615.473	488.877	4.157	3.302
PPL_05760	6.582	27.317	31.378	4.150	4.767
PPL_05171	27.009	111.901	40.463	4.143	1.498
PPL_07774	0.372	1.539	1.506	4.142	4.053
PPL_06343	0.643	2.664	2.316	4.140	3.599
PPL_01978	1.666	6.890	5.374	4.135	3.225
PPL_03722	2.532	10.466	7.586	4.133	2.996
PPL_10628	1.384	5.719	4.823	4.133	3.486
PPL_00615	0.078	0.321	0.506	4.128	6.502
PPL_01920	2.079	8.579	6.627	4.127	3.188
PPL_00650	2.931	12.060	11.059	4.115	3.774
PPL_01609	2.278	9.364	6.076	4.111	2.668
PPL_05043	0.827	3.400	2.771	4.110	3.350
PPL_05593	1.960	8.038	7.534	4.101	3.844
PPL_02319	5.709	23.377	23.955	4.095	4.196
PPL_03563	0.369	1.510	1.396	4.091	3.782
PPL_09526	0.384	1.568	1.212	4.087	3.159
PPL_07630	1.055	4.304	5.420	4.081	5.139
PPL_11382	1.328	5.407	6.978	4.071	5.254
PPL_02487	4.123	16.772	13.595	4.068	3.297
PPL_09971	5.097	20.731	16.562	4.067	3.249
PPL_11099	2.054	8.337	6.970	4.060	3.394
PPL_11778	0.601	2.438	1.658	4.058	2.760
PPL_01610	5.309	21.526	19.624	4.055	3.696

PPL_04402	0.359	1.453	1.055	4.043	2.934
PPL_02947	1.201	4.852	3.903	4.041	3.250
PPL_12586	0.141	0.571	0.384	4.036	2.717
PPL_12224	144.267	581.429	679.912	4.030	4.713
PPL_04955	0.414	1.667	1.488	4.029	3.595
PPL_00903	0.397	1.599	0.500	4.028	1.260
PPL_11598	37.083	149.230	77.682	4.024	2.095
PPL_08197	0.311	1.249	1.626	4.021	5.234
PPL_10765	27.920	112.175	52.052	4.018	1.864
PPL_11396	1.227	4.923	4.534	4.013	3.696
PPL_07389	1.111	4.457	2.893	4.013	2.605
PPL_01596	4.572	18.306	15.516	4.004	3.394
PPL_02478	5.575	22.309	11.744	4.002	2.107
PPL_03001	3.264	13.043	14.816	3.996	4.539
PPL_02736	1.355	5.416	3.658	3.996	2.699
PPL_10562	4.384	17.509	13.905	3.994	3.172
PPL_09637	2.307	9.200	3.662	3.989	1.588
PPL_05074	6.057	24.130	21.405	3.984	3.534
PPL_05491	0.274	1.091	0.441	3.981	1.609
PPL_04261	0.891	3.543	3.651	3.975	4.095
PPL_12367	5.009	19.911	18.489	3.975	3.691
PPL_06209	23.416	93.080	128.624	3.975	5.493
PPL_01093	11.988	47.614	49.035	3.972	4.090
PPL_04721	6.233	24.745	19.904	3.970	3.193
PPL_11894	15.465	61.370	58.128	3.968	3.759
PPL_07922	6.324	25.082	13.887	3.966	2.196
PPL_08310	2.438	9.658	7.919	3.961	3.248
PPL_12316	0.591	2.339	2.222	3.957	3.760
PPL_00876	0.643	2.544	2.406	3.955	3.741
PPL_03476	40.402	159.610	56.185	3.951	1.391
PPL_11509	4.065	16.051	14.303	3.949	3.519
PPL_07670	19.697	77.764	80.870	3.948	4.106
PPL_01573	5.419	21.391	19.540	3.947	3.606
PPL_06961	11.440	45.156	27.371	3.947	2.393
PPL_08052	0.315	1.239	0.858	3.939	2.728
PPL_05012	22.964	90.386	80.816	3.936	3.519
PPL_02046	18.554	72.891	143.320	3.929	7.724
PPL_09081	1.050	4.121	3.041	3.925	2.896
PPL_10119	3.380	13.264	10.193	3.925	3.016
PPL_03053	14.664	57.548	58.483	3.925	3.988
PPL_08668	12.032	47.213	16.197	3.924	1.346
PPL_04938	0.223	0.876	0.627	3.922	2.806
PPL_06153	0.939	3.682	1.813	3.920	1.930
PPL_06116	4.826	18.915	19.131	3.920	3.964
PPL_06393	0.305	1.193	1.346	3.915	4.417
PPL_08353	0.123	0.482	0.349	3.911	2.832
PPL_04418	0.486	1.899	2.234	3.911	4.601
PPL_01907	0.397	1.553	1.252	3.911	3.152
PPL_05998	2.106	8.232	8.296	3.908	3.938
PPL_05584	15.002	58.570	58.570	3.904	3.904
PPL_03971	1.135	4.427	4.010	3.901	3.534
PPL_07688	1.907	7.437	7.027	3.900	3.685

PPL_00229	0.319	1.246	1.282	3.900	4.012
PPL_12361	19.627	76.501	55.975	3.898	2.852
PPL_03616	12.420	48.343	19.610	3.892	1.579
PPL_09169	0.858	3.339	2.113	3.892	2.463
PPL_10789	0.255	0.990	0.448	3.881	1.756
PPL_06318	2.723	10.565	12.107	3.880	4.446
PPL_01516	5.148	19.971	16.715	3.880	3.247
PPL_08475	1.633	6.333	10.241	3.879	6.272
PPL_02944	2.329	9.033	6.943	3.878	2.980
PPL_06311	594.066	2302.449	1414.487	3.876	2.381
PPL_11519	6.804	26.331	35.926	3.870	5.280
PPL_01274	5.208	20.137	16.022	3.866	3.076
PPL_07778	0.852	3.284	1.723	3.854	2.022
PPL_03028	0.390	1.502	0.192	3.854	0.493
PPL_08166	64.029	246.311	230.957	3.847	3.607
PPL_10268	2.233	8.585	6.751	3.844	3.023
PPL_05062	0.190	0.731	0.687	3.842	3.613
PPL_02171	0.327	1.255	1.122	3.840	3.434
PPL_03464	1.068	4.097	3.681	3.835	3.445
PPL_00065	1107.776	4243.324	3878.237	3.830	3.501
PPL_00720	0.062	0.236	0.219	3.827	3.537
PPL_00873	2.364	9.045	4.823	3.826	2.040
PPL_07890	0.219	0.839	2.461	3.825	11.220
PPL_12512	1.111	4.247	3.119	3.824	2.808
PPL_08444	0.268	1.024	0.827	3.821	3.087
PPL_04396	0.423	1.616	1.269	3.820	3.000
PPL_08641	2.649	10.109	14.641	3.816	5.528
PPL_09663	6.149	23.462	15.080	3.815	2.452
PPL_12435	4.828	18.416	18.643	3.814	3.861
PPL_08745	2.327	8.870	10.274	3.811	4.415
PPL_03885	1.373	5.234	3.420	3.811	2.491
PPL_03784	0.270	1.026	1.176	3.806	4.363
PPL_12329	1.614	6.137	5.894	3.802	3.651
PPL_06761	9.633	36.558	41.699	3.795	4.329
PPL_03090	6.352	24.072	18.973	3.789	2.987
PPL_04162	0.813	3.078	3.363	3.787	4.137
PPL_07674	4.190	15.864	14.925	3.786	3.562
PPL_04223	0.633	2.391	2.160	3.780	3.416
PPL_00073	1.655	6.254	4.955	3.779	2.994
PPL_08640	2.595	9.788	8.234	3.772	3.173
PPL_09773	0.295	1.113	0.881	3.769	2.983
PPL_11397	0.155	0.585	0.357	3.767	2.299
PPL_06886	1.577	5.938	5.581	3.766	3.540
PPL_08645	15.975	60.154	54.364	3.765	3.403
PPL_08793	2.009	7.561	5.487	3.764	2.732
PPL_06691	2.689	10.122	11.747	3.764	4.368
PPL_00718	0.903	3.399	2.949	3.764	3.266
PPL_09537	2.028	7.623	3.277	3.758	1.616
PPL_09182	2.930	11.001	13.423	3.754	4.581
PPL_07149	0.702	2.635	2.684	3.751	3.822
PPL_11066	9.245	34.669	22.027	3.750	2.382
PPL_01911	2.180	8.171	5.828	3.749	2.674

PPL_00922	0.071	0.267	0.225	3.734	3.149
PPL_08012	0.073	0.272	0.183	3.734	2.520
PPL_00968	0.084	0.313	0.527	3.734	6.301
PPL_11820	4.543	16.938	19.878	3.728	4.376
PPL_01257	1.603	5.968	4.721	3.724	2.946
PPL_07002	12.497	46.517	38.001	3.722	3.041
PPL_05003	8.928	33.198	18.415	3.718	2.063
PPL_07477	5.515	20.495	15.975	3.716	2.897
PPL_09742	8.958	33.281	28.305	3.715	3.160
PPL_02297	1.244	4.622	3.360	3.714	2.700
PPL_10709	0.861	3.196	2.555	3.714	2.969
PPL_08108	4.380	16.256	9.102	3.712	2.078
PPL_04419	0.665	2.463	1.329	3.707	2.000
PPL_00961	1.149	4.259	3.030	3.707	2.637
PPL_04933	0.458	1.695	1.248	3.703	2.725
PPL_10815	11.563	42.722	47.855	3.695	4.139
PPL_10323	0.348	1.282	1.241	3.690	3.569
PPL_08589	0.285	1.052	0.881	3.689	3.089
PPL_12293	7.592	28.002	24.701	3.688	3.254
PPL_11105	56.649	208.873	173.776	3.687	3.068
PPL_02191	4.222	15.560	16.677	3.686	3.950
PPL_07932	8.235	30.350	33.562	3.686	4.076
PPL_07214	0.433	1.594	1.511	3.683	3.491
PPL_00656	0.618	2.275	1.651	3.683	2.673
PPL_06800	0.875	3.221	2.603	3.682	2.976
PPL_01192	7.375	27.131	20.464	3.679	2.775
PPL_10535	24.831	91.272	85.423	3.676	3.440
PPL_02025	1.948	7.157	5.094	3.674	2.615
PPL_06895	0.327	1.202	0.980	3.671	2.993
PPL_09472	0.094	0.344	0.340	3.671	3.624
PPL_01779	20.836	76.477	34.929	3.670	1.676
PPL_01045	10.686	39.181	45.935	3.667	4.299
PPL_07345	4.215	15.452	16.196	3.666	3.842
PPL_01811	7.500	27.487	19.715	3.665	2.629
PPL_08189	5.244	19.218	14.464	3.664	2.758
PPL_09150	2.467	9.032	4.876	3.661	1.976
PPL_02258	1.360	4.978	5.528	3.661	4.065
PPL_01821	0.777	2.841	2.103	3.658	2.708
PPL_12460	3.289	12.024	11.270	3.656	3.427
PPL_08168	2.599	9.485	12.138	3.650	4.670
PPL_07223	37.207	135.788	50.895	3.650	1.368
PPL_01541	69.451	253.346	223.146	3.648	3.213
PPL_01662	5.043	18.383	26.757	3.646	5.306
PPL_06270	0.603	2.196	0.851	3.641	1.410
PPL_12185	1.282	4.662	3.754	3.636	2.927
PPL_01858	71.886	261.378	376.187	3.636	5.233
PPL_02690	10.775	39.167	45.989	3.635	4.268
PPL_04019	2.748	9.989	8.255	3.635	3.004
PPL_04535	5.439	19.733	15.999	3.628	2.941
PPL_09202	0.279	1.011	1.039	3.626	3.728
PPL_02584	0.723	2.621	1.186	3.625	1.640
PPL_00819	3.439	12.461	11.126	3.624	3.236

PPL_02544	0.368	1.335	1.272	3.623	3.451
PPL_08862	0.291	1.053	0.688	3.620	2.366
PPL_00360	0.935	3.384	3.092	3.619	3.307
PPL_09365	1.435	5.191	4.900	3.617	3.414
PPL_07207	15.235	55.083	79.112	3.615	5.193
PPL_02027	2.849	10.291	8.844	3.612	3.104
PPL_09046	20.164	72.641	82.660	3.602	4.099
PPL_04213	5.601	20.168	13.144	3.601	2.347
PPL_02235	4.038	14.517	17.005	3.595	4.211
PPL_12390	9.496	34.144	31.312	3.595	3.297
PPL_03753	12.232	43.940	37.051	3.592	3.029
PPL_04781	16.158	57.965	31.210	3.587	1.932
PPL_12570	15.278	54.776	47.990	3.585	3.141
PPL_06074	0.110	0.395	0.300	3.581	2.715
PPL_05682	1.714	6.119	5.595	3.570	3.264
PPL_00316	9.698	34.620	30.484	3.570	3.143
PPL_01170	1.344	4.796	3.747	3.567	2.787
PPL_10693	0.517	1.844	0.970	3.564	1.875
PPL_03956	5.716	20.349	20.308	3.560	3.553
PPL_07557	2.739	9.746	2.833	3.559	1.034
PPL_00025	0.134	0.475	0.368	3.557	2.755
PPL_09053	0.735	2.613	2.631	3.557	3.582
PPL_05710	4.933	17.549	9.768	3.557	1.980
PPL_11119	42.643	151.539	165.318	3.554	3.877
PPL_10154	0.796	2.825	2.602	3.549	3.268
PPL_01560	12.677	44.959	50.166	3.546	3.957
PPL_05963	10.326	36.562	21.497	3.541	2.082
PPL_09131	7.843	27.740	22.732	3.537	2.898
PPL_11663	0.082	0.289	0.353	3.533	4.314
PPL_03233	4.814	16.967	15.222	3.525	3.162
PPL_02952	3.618	12.736	12.974	3.520	3.586
PPL_08752	49.993	175.793	80.167	3.516	1.604
PPL_10299	1.551	5.451	2.618	3.514	1.687
PPL_09769	0.774	2.720	1.438	3.514	1.858
PPL_11208	15.705	54.972	63.982	3.500	4.074
PPL_04752	0.367	1.283	1.751	3.500	4.777
PPL_08836	0.197	0.688	0.825	3.486	4.183
PPL_05659	0.513	1.787	1.233	3.481	2.402
PPL_05029	1.144	3.968	5.244	3.469	4.584
PPL_07451	1.085	3.762	3.776	3.468	3.481
PPL_03774	1.534	5.314	4.174	3.463	2.720
PPL_04443	5.221	18.036	11.583	3.455	2.219
PPL_02786	440.372	1521.141	1132.616	3.454	2.572
PPL_09607	1.526	5.267	4.028	3.452	2.640
PPL_09448	46.686	161.064	80.356	3.450	1.721
PPL_10057	12.880	44.383	31.356	3.446	2.434
PPL_05502	6.182	21.301	15.724	3.446	2.544
PPL_07855	66.108	227.600	261.176	3.443	3.951
PPL_04697	1.044	3.592	2.073	3.440	1.986
PPL_05363	9.251	31.817	23.949	3.439	2.589
PPL_05546	1.036	3.561	1.955	3.437	1.886
PPL_07946	10.041	34.480	40.139	3.434	3.998

PPL_05453	2.215	7.603	6.404	3.432	2.891
PPL_07399	2.559	8.780	5.539	3.431	2.164
PPL_01064	3.762	12.893	5.355	3.427	1.423
PPL_03355	5.415	18.554	21.183	3.427	3.912
PPL_03224	0.537	1.840	1.324	3.426	2.465
PPL_03173	6.593	22.496	19.062	3.412	2.891
PPL_01845	0.739	2.521	1.968	3.411	2.663
PPL_09727	2.219	7.546	6.978	3.401	3.145
PPL_03951	7.031	23.840	28.073	3.391	3.993
PPL_06054	14.487	49.050	48.939	3.386	3.378
PPL_09170	0.913	3.087	2.334	3.380	2.556
PPL_05274	3.212	10.856	8.890	3.380	2.768
PPL_09462	0.704	2.374	1.898	3.372	2.695
PPL_11260	39.498	132.789	140.239	3.362	3.551
PPL_09091	2.289	7.692	6.283	3.360	2.744
PPL_05357	2.354	7.902	6.870	3.357	2.918
PPL_11717	36.916	123.183	78.201	3.337	2.118
PPL_10380	0.726	2.422	3.211	3.335	4.422
PPL_10721	81.588	271.376	220.033	3.326	2.697
PPL_11463	6.736	22.312	15.094	3.312	2.241
PPL_03897	16.230	53.662	34.445	3.306	2.122
PPL_10120	0.536	1.770	1.228	3.305	2.292
PPL_02015	13.649	45.099	59.495	3.304	4.359
PPL_07406	0.738	2.439	2.645	3.304	3.583
PPL_02743	1.194	3.945	3.674	3.303	3.076
PPL_01512	1.314	4.339	3.013	3.302	2.293
PPL_01555	8.376	27.652	20.472	3.301	2.444
PPL_05439	3.546	11.689	8.321	3.297	2.347
PPL_03411	0.584	1.925	1.355	3.297	2.321
PPL_09477	0.589	1.942	1.382	3.297	2.346
PPL_01818	5.201	17.142	14.029	3.296	2.697
PPL_03163	1.666	5.487	4.397	3.294	2.640
PPL_04195	69.570	229.084	320.783	3.293	4.611
PPL_07143	3.425	11.273	13.508	3.291	3.944
PPL_10854	5.396	17.733	17.693	3.286	3.279
PPL_11168	0.394	1.294	1.071	3.281	2.716
PPL_09290	10.537	34.538	21.737	3.278	2.063
PPL_07659	8.538	27.914	53.532	3.269	6.270
PPL_00182	13.161	42.973	27.071	3.265	2.057
PPL_00786	0.535	1.747	1.170	3.265	2.186
PPL_03112	1.734	5.660	6.148	3.265	3.546
PPL_04262	76.863	250.901	333.020	3.264	4.333
PPL_10652	2.387	7.775	6.330	3.257	2.652
PPL_03188	4.456	14.511	10.165	3.256	2.281
PPL_01530	0.919	2.987	2.687	3.250	2.924
PPL_00277	12.384	40.204	22.967	3.246	1.855
PPL_08557	1.261	4.082	3.261	3.238	2.586
PPL_01525	1.205	3.898	3.065	3.236	2.544
PPL_01389	0.690	2.230	1.651	3.233	2.394
PPL_00356	3.644	11.740	8.842	3.222	2.426
PPL_07412	3.494	11.239	8.676	3.217	2.483
PPL_05829	20.288	65.245	53.788	3.216	2.651

PPL_06698	4.380	14.057	14.105	3.209	3.220
PPL_12565	5.409	17.346	16.474	3.207	3.046
PPL_05964	1.584	5.076	3.987	3.204	2.517
PPL_04797	11.319	36.232	23.351	3.201	2.063
PPL_10447	9.908	31.622	30.801	3.192	3.109
PPL_01065	1.683	5.372	3.710	3.192	2.204
PPL_05947	3.261	10.395	8.817	3.187	2.704
PPL_04346	0.287	0.915	0.774	3.187	2.696
PPL_09294	17.810	56.758	29.824	3.187	1.675
PPL_12364	0.418	1.332	0.816	3.184	1.950
PPL_00977	1.014	3.228	3.063	3.182	3.019
PPL_00649	23.584	74.911	61.803	3.176	2.621
PPL_09799	46.071	146.031	115.706	3.170	2.511
PPL_12416	2.904	9.151	6.503	3.151	2.239
PPL_00994	37.222	117.302	19.745	3.151	0.530
PPL_01834	0.769	2.413	2.025	3.139	2.634
PPL_10895	11.991	37.609	18.453	3.136	1.539
PPL_11686	0.683	2.141	1.726	3.133	2.525
PPL_04521	4.488	14.030	10.876	3.126	2.424
PPL_03562	2.614	8.148	10.465	3.117	4.003
PPL_09516	13.189	41.112	32.625	3.117	2.474
PPL_01420	1.504	4.679	3.016	3.112	2.005
PPL_09791	41.988	130.559	108.103	3.109	2.575
PPL_02940	1.169	3.625	3.370	3.102	2.884
PPL_01181	6.504	20.162	14.502	3.100	2.230
PPL_12303	9.050	27.999	28.869	3.094	3.190
PPL_00914	162.703	502.224	202.500	3.087	1.245
PPL_00728	2.388	7.365	5.853	3.085	2.451
PPL_05338	1.127	3.463	4.119	3.074	3.655
PPL_10801	2.124	6.498	6.696	3.060	3.153
PPL_08764	39.120	119.435	117.984	3.053	3.016
PPL_03179	1.023	3.108	6.158	3.040	6.022
PPL_11365	54.307	164.479	138.565	3.029	2.552
PPL_07859	122.218	39.799	77.368	0.326	0.633
PPL_00995	68.612	22.239	21.447	0.324	0.313
PPL_11728	65.731	21.219	15.186	0.323	0.231
PPL_12602	0.491	0.158	0.099	0.322	0.201
PPL_00871	1517.455	482.175	319.570	0.318	0.211
PPL_01136	30.778	9.774	8.057	0.318	0.262
PPL_09190	1094.476	345.486	319.642	0.316	0.292
PPL_06073	41.577	13.101	11.290	0.315	0.272
PPL_06462	12.207	3.831	5.119	0.314	0.419
PPL_00267	41.460	12.934	12.218	0.312	0.295
PPL_09564	31.736	9.859	10.958	0.311	0.345
PPL_06960	19.594	6.026	9.951	0.308	0.508
PPL_11489	232.983	71.796	79.511	0.308	0.341
PPL_03260	264.638	81.631	82.869	0.308	0.313
PPL_03534	28.626	8.803	15.697	0.308	0.548
PPL_02513	131.881	40.438	38.037	0.307	0.288
PPL_06940	144.822	44.212	40.690	0.305	0.281
PPL_06161	137.859	42.023	31.560	0.305	0.229
PPL_07328	13.859	4.193	5.582	0.303	0.403

PPL_11171	64.661	19.474	30.459	0.301	0.471
PPL_08205	3.042	0.914	0.859	0.301	0.282
PPL_08601	57.324	17.179	16.812	0.300	0.293
PPL_11059	26.396	7.923	2.476	0.300	0.094
PPL_03695	160.282	48.035	34.668	0.300	0.216
PPL_11303	8.406	2.515	4.706	0.299	0.560
PPL_01924	52.183	15.512	13.704	0.297	0.263
PPL_03891	241.581	71.449	30.393	0.296	0.126
PPL_06741	127.081	37.657	39.373	0.296	0.310
PPL_09463	22.121	6.542	7.000	0.296	0.316
PPL_00478	34.383	10.144	9.557	0.295	0.278
PPL_12212	19.458	5.722	6.931	0.294	0.356
PPL_02831	0.604	0.177	0.269	0.293	0.445
PPL_12112	16.592	4.860	8.568	0.293	0.516
PPL_05061	22.727	6.605	7.254	0.291	0.319
PPL_03988	66.203	19.168	38.925	0.290	0.588
PPL_02987	80.716	23.308	27.544	0.289	0.341
PPL_00439	29.186	8.397	8.297	0.288	0.284
PPL_04183	12.259	3.535	5.355	0.288	0.437
PPL_06356	108.012	31.089	42.015	0.288	0.389
PPL_09400	4.100	1.177	1.243	0.287	0.303
PPL_07051	141.394	40.646	36.604	0.287	0.259
PPL_02419	5.582	1.600	7.554	0.287	1.353
PPL_04168	0.224	0.064	0.100	0.286	0.448
PPL_07752	7.525	2.155	3.337	0.286	0.443
PPL_10240	22.039	6.301	6.805	0.286	0.309
PPL_04225	105.262	29.758	35.788	0.283	0.340
PPL_05121	15.852	4.478	11.091	0.283	0.700
PPL_10465	7.886	2.220	3.899	0.282	0.494
PPL_08209	10.966	3.067	2.541	0.280	0.232
PPL_01355	23.940	6.712	9.997	0.280	0.418
PPL_06007	2.810	0.785	0.947	0.279	0.337
PPL_04408	45.822	12.718	15.812	0.278	0.345
PPL_01187	4.076	1.129	1.345	0.277	0.330
PPL_09811	26.579	7.300	5.257	0.275	0.198
PPL_05109	49.065	13.517	9.863	0.275	0.201
PPL_03567	4.191	1.154	0.640	0.275	0.153
PPL_10616	77.209	21.066	30.481	0.273	0.395
PPL_00191	31.366	8.570	11.078	0.273	0.353
PPL_07822	16.621	4.504	3.196	0.271	0.192
PPL_11949	46.545	12.594	14.390	0.271	0.309
PPL_03954	72.618	19.572	18.545	0.270	0.255
PPL_11060	25.606	6.918	2.707	0.270	0.106
PPL_09721	2.877	0.775	0.442	0.269	0.154
PPL_10011	54.287	14.528	12.922	0.268	0.238
PPL_04616	11.020	2.946	2.926	0.267	0.265
PPL_05952	24.650	6.552	7.174	0.266	0.291
PPL_04910	66.362	17.592	14.299	0.265	0.215
PPL_03350	15.844	4.191	4.997	0.265	0.315
PPL_07539	18.761	4.927	5.078	0.263	0.271
PPL_06722	21.643	5.700	5.810	0.263	0.268
PPL_02238	103.006	27.082	26.527	0.263	0.258

PPL_09257	10.030	2.627	2.773	0.262	0.277
PPL_10130	108.505	28.459	25.044	0.262	0.231
PPL_09126	59.840	15.603	18.192	0.261	0.304
PPL_09247	10.277	2.678	2.741	0.261	0.267
PPL_12107	7.214	1.873	2.071	0.260	0.287
PPL_04587	10.452	2.722	2.970	0.260	0.284
PPL_05615	9.869	2.552	3.200	0.259	0.324
PPL_03782	69.632	17.960	27.893	0.258	0.401
PPL_09097	0.148	0.038	0.055	0.258	0.375
PPL_11476	36.136	9.326	11.842	0.258	0.328
PPL_10036	15.744	4.048	3.764	0.257	0.239
PPL_10504	3.818	0.978	1.327	0.256	0.348
PPL_10884	21.261	5.441	5.936	0.256	0.279
PPL_02798	16.758	4.280	5.415	0.255	0.323
PPL_01544	88.059	22.442	27.100	0.255	0.308
PPL_00653	18.000	4.566	3.295	0.254	0.183
PPL_02322	3.949	1.001	1.012	0.253	0.256
PPL_11272	9.362	2.371	2.327	0.253	0.249
PPL_06915	142.854	35.952	36.008	0.252	0.252
PPL_01502	8.418	2.107	3.660	0.250	0.435
PPL_11277	44.349	11.074	15.550	0.250	0.351
PPL_07800	7.689	1.909	3.788	0.248	0.493
PPL_11784	44.601	11.071	16.910	0.248	0.379
PPL_02508	6.234	1.538	1.349	0.247	0.216
PPL_04902	2.084	0.512	1.032	0.246	0.495
PPL_01110	51.411	12.579	13.924	0.245	0.271
PPL_09020	0.510	0.124	0.120	0.244	0.236
PPL_01959	90.831	22.198	18.781	0.244	0.207
PPL_03739	26.842	6.562	2.826	0.244	0.105
PPL_04525	49.299	11.982	15.987	0.243	0.324
PPL_01159	8.344	2.028	1.282	0.243	0.154
PPL_12334	108.530	25.957	34.957	0.239	0.322
PPL_00630	47.256	11.271	15.261	0.239	0.323
PPL_05727	59.170	14.165	18.866	0.239	0.319
PPL_00735	63.217	15.036	18.239	0.238	0.289
PPL_03578	66.777	15.901	14.518	0.238	0.217
PPL_06453	165.051	39.207	52.353	0.238	0.317
PPL_10008	100.002	23.784	22.715	0.238	0.227
PPL_06710	147.869	35.128	43.361	0.238	0.293
PPL_12467	168.390	39.676	30.855	0.236	0.183
PPL_07318	26.866	6.340	7.334	0.236	0.273
PPL_02100	36.590	8.614	9.804	0.235	0.268
PPL_11411	1.632	0.384	0.597	0.235	0.366
PPL_02110	167.508	39.396	53.837	0.235	0.321
PPL_09641	18.972	4.466	6.343	0.235	0.334
PPL_10181	67.257	15.772	12.961	0.235	0.193
PPL_04384	8.610	2.011	2.627	0.234	0.305
PPL_05551	70.456	16.455	22.004	0.234	0.312
PPL_09181	71.737	16.787	19.069	0.234	0.266
PPL_03898	41.428	9.645	10.113	0.233	0.244
PPL_07163	62.214	14.472	17.128	0.233	0.275
PPL_09729	19.128	4.441	5.932	0.232	0.310

PPL_00904	35.080	8.153	10.570	0.232	0.301
PPL_05418	55.227	12.828	7.946	0.232	0.144
PPL_00166	34.098	7.914	7.047	0.232	0.207
PPL_08485	2.256	0.522	0.486	0.231	0.215
PPL_03547	85.216	19.645	15.452	0.231	0.181
PPL_08137	10.702	2.457	1.608	0.230	0.150
PPL_06004	42.355	9.758	8.414	0.230	0.199
PPL_08487	21.355	4.916	3.213	0.230	0.150
PPL_06709	39.473	9.009	11.533	0.228	0.292
PPL_08863	2.710	0.616	0.711	0.227	0.262
PPL_10206	5.269	1.192	1.221	0.226	0.232
PPL_06742	35.483	8.036	7.258	0.226	0.205
PPL_07175	11.364	2.553	7.495	0.225	0.660
PPL_08034	34.619	7.781	14.006	0.225	0.405
PPL_05978	0.266	0.060	0.116	0.225	0.436
PPL_11813	6.546	1.474	1.673	0.225	0.255
PPL_04492	3.095	0.689	0.732	0.223	0.236
PPL_10647	22.314	4.983	3.756	0.223	0.168
PPL_08207	6.207	1.378	1.244	0.222	0.200
PPL_09237	22.069	4.886	6.760	0.221	0.306
PPL_07812	1.379	0.302	0.795	0.219	0.576
PPL_08516	1009.684	221.210	78.544	0.219	0.078
PPL_07305	72.802	15.964	26.143	0.219	0.359
PPL_00222	248.940	54.533	102.483	0.219	0.412
PPL_10096	4.690	1.022	0.869	0.218	0.185
PPL_08101	4.742	1.036	2.565	0.218	0.541
PPL_05060	13.205	2.872	4.887	0.217	0.370
PPL_01252	268.320	58.152	57.574	0.217	0.215
PPL_03962	39.845	8.623	13.927	0.216	0.350
PPL_08293	12.945	2.756	5.269	0.213	0.407
PPL_12166	5.949	1.269	0.956	0.213	0.161
PPL_01032	153.398	32.525	30.928	0.212	0.202
PPL_05630	59.991	12.664	17.677	0.211	0.295
PPL_07593	2.116	0.447	0.145	0.211	0.069
PPL_11385	27.007	5.679	10.675	0.210	0.395
PPL_02067	72.753	15.286	17.726	0.210	0.244
PPL_02412	66.891	14.075	22.711	0.210	0.340
PPL_09829	105.889	22.252	27.169	0.210	0.257
PPL_04224	11.303	2.368	3.596	0.209	0.318
PPL_06666	18.189	3.782	3.033	0.208	0.167
PPL_00295	6.881	1.430	0.985	0.208	0.143
PPL_08542	37.165	7.739	8.201	0.208	0.221
PPL_00488	65.875	13.650	13.798	0.207	0.209
PPL_00888	125.297	25.858	29.152	0.206	0.233
PPL_11661	261.972	53.855	49.410	0.206	0.189
PPL_07425	9.356	1.920	2.752	0.205	0.294
PPL_01927	46.770	9.553	8.569	0.204	0.183
PPL_00193	107.825	21.950	25.697	0.204	0.238
PPL_03640	1.163	0.236	0.327	0.203	0.281
PPL_01054	63.423	12.851	32.702	0.203	0.516
PPL_00647	109.879	22.260	37.022	0.203	0.337
PPL_01180	20.793	4.181	4.893	0.201	0.235

PPL_01600	23.011	4.618	2.077	0.201	0.090
PPL_04625	0.803	0.160	0.125	0.200	0.156
PPL_07584	22.159	4.442	4.730	0.200	0.213
PPL_07392	3.504	0.697	0.712	0.199	0.203
PPL_02437	6.518	1.288	0.750	0.198	0.115
PPL_11081	95.329	18.876	13.281	0.198	0.139
PPL_11129	3.082	0.606	0.513	0.197	0.166
PPL_06942	271.157	53.380	45.061	0.197	0.166
PPL_00489	91.685	18.101	35.337	0.197	0.385
PPL_05895	15.190	2.980	3.249	0.196	0.214
PPL_08057	25.008	4.905	2.781	0.196	0.111
PPL_08427	4.350	0.848	1.328	0.195	0.305
PPL_03914	273.744	53.144	125.752	0.194	0.459
PPL_06429	28.532	5.538	1.704	0.194	0.060
PPL_01095	56.316	10.878	13.493	0.193	0.240
PPL_08849	25.131	4.830	6.880	0.192	0.274
PPL_04561	3.577	0.688	1.008	0.192	0.282
PPL_02435	53.901	10.178	9.895	0.189	0.184
PPL_08928	99.289	18.680	16.476	0.188	0.166
PPL_00981	69.575	13.027	14.936	0.187	0.215
PPL_05689	179.976	33.537	41.255	0.186	0.229
PPL_09484	115.434	21.469	22.899	0.186	0.198
PPL_11091	1.677	0.311	0.335	0.185	0.200
PPL_07929	37.087	6.831	6.407	0.184	0.173
PPL_05205	7.660	1.403	1.806	0.183	0.236
PPL_12387	13.650	2.491	2.249	0.183	0.165
PPL_06849	27.053	4.941	6.935	0.183	0.256
PPL_01801	3.374	0.605	0.459	0.179	0.136
PPL_04602	11.934	2.135	1.507	0.179	0.126
PPL_00793	1.997	0.355	0.240	0.178	0.120
PPL_11461	150.915	26.920	22.982	0.178	0.152
PPL_05155	288.436	51.078	27.765	0.177	0.096
PPL_11092	6.956	1.224	1.706	0.176	0.245
PPL_07610	1.296	0.225	0.083	0.174	0.064
PPL_08595	4.840	0.843	1.285	0.174	0.265
PPL_05076	961.923	166.664	95.529	0.173	0.099
PPL_03913	344.785	59.645	158.553	0.173	0.460
PPL_10022	332.406	57.295	41.852	0.172	0.126
PPL_12388	6.595	1.132	1.264	0.172	0.192
PPL_10122	7.766	1.332	1.910	0.171	0.246
PPL_00279	27.630	4.726	7.198	0.171	0.260
PPL_02493	51.890	8.880	8.197	0.171	0.158
PPL_04669	4.880	0.836	0.927	0.171	0.190
PPL_05404	1.672	0.284	0.329	0.170	0.197
PPL_02791	164.277	27.933	20.111	0.170	0.122
PPL_05467	122.893	20.896	41.788	0.170	0.340
PPL_08575	74.280	12.636	17.893	0.170	0.241
PPL_00782	116.401	19.650	9.085	0.169	0.078
PPL_08711	0.855	0.143	0.163	0.168	0.191
PPL_03738	6.011	1.004	0.713	0.167	0.119
PPL_00690	1.519	0.254	0.341	0.167	0.224
PPL_00805	19.447	3.226	1.565	0.166	0.080

PPL_05148	0.456	0.075	0.165	0.165	0.362
PPL_12359	23.650	3.828	2.123	0.162	0.090
PPL_08815	31.060	5.025	6.424	0.162	0.207
PPL_11002	70.439	11.308	9.407	0.161	0.134
PPL_12127	255.371	40.747	56.948	0.160	0.223
PPL_00980	36.386	5.826	6.533	0.160	0.180
PPL_00407	32.716	5.178	6.243	0.158	0.191
PPL_03667	213.539	33.450	52.269	0.157	0.245
PPL_01379	30.787	4.840	1.251	0.157	0.041
PPL_02859	44.477	6.885	3.585	0.155	0.081
PPL_10279	35.216	5.468	5.894	0.155	0.167
PPL_05756	255.832	39.477	32.249	0.154	0.126
PPL_08161	137.548	21.070	78.505	0.153	0.571
PPL_08655	6.212	0.951	0.906	0.153	0.146
PPL_01644	0.792	0.121	0.234	0.153	0.296
PPL_05184	571.568	87.158	157.885	0.152	0.276
PPL_02250	184.639	27.473	12.307	0.149	0.067
PPL_11948	82.134	12.232	14.995	0.149	0.183
PPL_06585	31.771	4.697	5.905	0.148	0.186
PPL_09142	0.917	0.135	0.458	0.147	0.500
PPL_00624	20.558	3.002	8.037	0.146	0.391
PPL_01477	347.953	50.703	55.785	0.146	0.160
PPL_07801	0.534	0.078	0.163	0.146	0.305
PPL_09328	214.993	30.971	31.655	0.144	0.147
PPL_02317	19.222	2.751	1.421	0.143	0.074
PPL_04500	9.082	1.277	4.126	0.141	0.454
PPL_04484	35.458	4.979	2.529	0.140	0.071
PPL_07022	4.758	0.668	0.723	0.140	0.152
PPL_11023	120.871	16.890	15.702	0.140	0.130
PPL_07798	11.067	1.547	1.790	0.140	0.162
PPL_00990	118.193	16.463	84.900	0.139	0.718
PPL_02174	20.870	2.881	4.434	0.138	0.212
PPL_10055	17.850	2.460	5.016	0.138	0.281
PPL_04543	81.501	11.203	9.495	0.137	0.116
PPL_01369	0.277	0.038	0.129	0.137	0.465
PPL_09766	2.022	0.275	0.516	0.136	0.255
PPL_04393	38.150	5.199	7.545	0.136	0.198
PPL_05087	601.117	81.956	68.011	0.136	0.113
PPL_09250	59.187	7.995	8.859	0.135	0.150
PPL_11404	6.886	0.931	0.721	0.135	0.105
PPL_08816	221.274	29.368	47.199	0.133	0.213
PPL_02637	1034.609	137.301	52.098	0.133	0.050
PPL_06262	55.275	7.377	2.742	0.133	0.050
PPL_12106	16.179	2.136	1.637	0.132	0.101
PPL_05614	53.806	7.079	11.971	0.132	0.222
PPL_00979	30.880	4.036	5.290	0.131	0.171
PPL_12337	59.972	7.881	2.707	0.131	0.045
PPL_01753	47.033	6.131	7.761	0.130	0.165
PPL_01008	89.967	11.604	13.724	0.129	0.153
PPL_07248	2.544	0.328	0.583	0.129	0.229
PPL_05238	18.661	2.364	1.319	0.127	0.071
PPL_07756	2.864	0.363	0.495	0.127	0.173

PPL_09141	324.893	40.903	8.609	0.126	0.026
PPL_11316	25.814	3.255	4.367	0.126	0.169
PPL_08574	269.162	33.524	13.830	0.125	0.051
PPL_04380	164.159	20.295	9.973	0.124	0.061
PPL_07132	317.047	39.045	37.393	0.123	0.118
PPL_02514	23.053	2.844	3.299	0.123	0.143
PPL_01901	17.805	2.194	0.847	0.123	0.048
PPL_10113	107.697	13.130	11.446	0.122	0.106
PPL_02881	122.029	14.880	13.749	0.122	0.113
PPL_04461	120.410	14.690	15.496	0.122	0.129
PPL_00450	23.684	2.879	1.405	0.122	0.059
PPL_09155	12.198	1.494	1.301	0.122	0.107
PPL_08409	10.861	1.294	3.897	0.119	0.359
PPL_03719	43.350	5.180	6.150	0.119	0.142
PPL_05163	36.108	4.261	5.760	0.118	0.160
PPL_01135	33.539	3.941	4.218	0.118	0.126
PPL_09594	2.308	0.260	0.500	0.113	0.217
PPL_00861	5.291	0.594	2.834	0.112	0.536
PPL_09314	2.149	0.240	0.222	0.112	0.103
PPL_03152	45.460	5.028	5.940	0.111	0.131
PPL_01999	27.045	2.995	2.371	0.111	0.088
PPL_07109	7.296	0.811	0.850	0.111	0.117
PPL_05806	56.095	5.989	4.156	0.107	0.074
PPL_06967	47.132	5.042	4.895	0.107	0.104
PPL_07028	997.121	105.957	129.613	0.106	0.130
PPL_01698	10.846	1.144	0.494	0.106	0.046
PPL_09707	10.349	1.088	0.921	0.105	0.089
PPL_10755	3.958	0.416	0.406	0.105	0.102
PPL_09336	52.925	5.509	5.782	0.104	0.109
PPL_09983	0.864	0.090	0.271	0.104	0.314
PPL_05570	1.435	0.145	0.091	0.101	0.063
PPL_05986	98.675	9.958	11.883	0.101	0.120
PPL_00658	32.418	3.083	2.774	0.095	0.086
PPL_08149	249.095	23.719	27.246	0.095	0.109
PPL_04626	29.097	2.744	3.926	0.094	0.135
PPL_09422	13.238	1.240	0.776	0.094	0.059
PPL_02819	27.445	2.553	0.980	0.093	0.036
PPL_09333	4.474	0.408	0.283	0.091	0.063
PPL_08970	295.722	25.940	33.955	0.088	0.115
PPL_07356	103.099	9.023	11.699	0.088	0.113
PPL_12572	9.895	0.862	0.666	0.087	0.067
PPL_10553	75.388	6.582	4.168	0.087	0.055
PPL_08312	4.278	0.370	0.135	0.086	0.032
PPL_01189	45.386	3.920	6.326	0.086	0.139
PPL_02737	0.594	0.050	0.236	0.084	0.397
PPL_11554	82.077	6.801	9.229	0.083	0.112
PPL_00206	30.600	2.535	4.932	0.083	0.161
PPL_07247	28.969	2.365	3.839	0.082	0.133
PPL_04909	53.167	4.256	3.637	0.080	0.068
PPL_04573	455.579	36.114	293.448	0.079	0.644
PPL_00802	26.914	2.117	0.778	0.079	0.029
PPL_05009	76.203	5.987	7.603	0.079	0.100

PPL_06261	112.960	8.762	4.718	0.078	0.042
PPL_09489	26.633	2.069	1.780	0.078	0.067
PPL_07763	47.222	3.636	2.406	0.077	0.051
PPL_02187	7.760	0.598	1.448	0.077	0.187
PPL_06345	7.732	0.580	0.644	0.075	0.083
PPL_09886	21.007	1.580	2.326	0.075	0.111
PPL_06272	29.939	2.118	2.628	0.071	0.088
PPL_08175	41.067	2.810	3.930	0.068	0.096
PPL_06743	11.101	0.759	0.496	0.068	0.045
PPL_05745	47.757	3.209	2.502	0.067	0.052
PPL_05149	20.800	1.398	5.051	0.067	0.243
PPL_01689	77.977	5.176	5.602	0.066	0.072
PPL_04562	66.250	4.405	7.438	0.066	0.112
PPL_10596	58.794	3.807	1.981	0.065	0.034
PPL_01967	29.809	1.858	4.345	0.062	0.146
PPL_02149	54.814	3.415	5.031	0.062	0.092
PPL_10446	1.901	0.117	0.183	0.061	0.096
PPL_10174	314.777	18.843	22.980	0.060	0.073
PPL_11723	115.405	6.663	1.749	0.058	0.015
PPL_07244	136.905	7.725	11.207	0.056	0.082
PPL_10668	53.377	2.957	2.444	0.055	0.046
PPL_12418	9.741	0.526	0.478	0.054	0.049
PPL_09184	51.382	2.647	6.428	0.052	0.125
PPL_11589	7.587	0.394	1.355	0.052	0.179
PPL_07385	178.764	8.845	7.351	0.049	0.041
PPL_10987	238.076	10.848	13.687	0.046	0.057
PPL_00127	15.616	0.713	1.105	0.046	0.071
PPL_00470	13.580	0.594	0.591	0.044	0.043
PPL_03581	8.791	0.366	2.313	0.042	0.263
PPL_07439	56.161	2.292	1.765	0.041	0.031
PPL_05162	270.354	11.110	5.971	0.041	0.022
PPL_08455	8.588	0.338	0.533	0.039	0.062
PPL_07438	68.451	2.537	3.106	0.037	0.045
PPL_08204	709.426	24.726	46.073	0.035	0.065
PPL_04929	36.297	1.280	1.032	0.035	0.028
PPL_09115	150.287	4.867	2.682	0.032	0.018
PPL_06587	7.823	0.244	0.176	0.031	0.022
PPL_08654	82.353	2.519	7.757	0.031	0.094
PPL_12417	25.504	0.758	1.295	0.030	0.051
PPL_10511	35.724	1.078	0.882	0.030	0.025
PPL_06854	11.664	0.334	0.665	0.029	0.057
PPL_07432	80.523	2.295	5.369	0.029	0.067
PPL_00173	37.522	0.986	2.404	0.026	0.064
PPL_07303	61.442	1.546	7.047	0.025	0.115
PPL_05815	10.174	0.258	0.250	0.025	0.025
PPL_06712	68.647	1.589	1.672	0.023	0.024
PPL_01859	22.737	0.467	0.774	0.021	0.034
PPL_09019	15.259	0.312	0.686	0.020	0.045
PPL_07729	61.076	1.231	4.790	0.020	0.078
PPL_10669	122.081	2.435	3.067	0.020	0.025
PPL_06819	4.443	0.089	0.083	0.020	0.019
PPL_06963	219.178	4.207	5.062	0.019	0.023

PPL_11179	30.275	0.412	0.555	0.014	0.018
PPL_06714	36.806	0.526	0.526	0.014	0.014
PPL_09988	255.404	2.519	6.523	0.010	0.026
PPL_06545	53.452	0.453	1.013	0.008	0.019
PPL_07847	420.482	0.653	0.513	0.002	0.001

Table A4: List of genes regulated by glorin after *P. pallidum* PN500 cells prestarved for 1 hr were stimulated with glorin for an additional 1 hr.

Gene ID	RPKM untreated	RPKM treated	Fold change treated/untreated
PPL_05354	17.359	987.834	56.907
PPL_09347	0.497	26.789	53.915
PPL_12248	4.010	159.175	39.699
PPL_12249	16.904	494.221	29.237
PPL_11763	0.927	21.002	22.667
PPL_03541	0.112	2.488	22.255
PPL_05833	3.441	72.021	20.930
PPL_06644	0.500	8.704	17.404
PPL_07811	0.332	5.444	16.374
PPL_08454	0.376	5.963	15.866
PPL_07812	0.302	4.675	15.490
PPL_00912	0.811	12.366	15.239
PPL_00117	93.761	1137.095	12.128
PPL_08455	0.338	4.051	11.983
PPL_00861	0.594	6.916	11.641
PPL_10324	3.096	35.963	11.616
PPL_04784	0.052	0.558	10.817
PPL_02621	25.605	239.969	9.372
PPL_02620	353.455	3300.083	9.337
PPL_12251	1.603	14.048	8.763
PPL_03564	3.124	27.046	8.656
PPL_07801	0.078	0.622	7.998
PPL_12271	11.361	87.249	7.680
PPL_04587	2.722	20.715	7.610
PPL_07296	21.092	155.598	7.377
PPL_07818	3.355	24.269	7.235
PPL_11303	2.515	17.436	6.932
PPL_07817	10.126	66.569	6.574
PPL_08496	5.374	34.480	6.416
PPL_00973	1.829	11.723	6.411
PPL_12308	20.420	129.378	6.336
PPL_12354	37.101	234.929	6.332
PPL_05452	69.272	402.077	5.804
PPL_08545	0.733	4.184	5.708
PPL_07800	1.909	10.474	5.487
PPL_03784	1.026	5.620	5.478
PPL_05392	0.827	4.362	5.273
PPL_07610	0.225	1.156	5.129
PPL_01618	5.703	29.001	5.085
PPL_07302	32.080	157.407	4.907
PPL_05390	0.144	0.700	4.868
PPL_03785	0.440	2.143	4.865
PPL_12250	5.091	24.279	4.769
PPL_10511	1.078	5.100	4.730

PPL_10193	0.279	1.289	4.625
PPL_10669	2.435	11.081	4.551
PPL_01544	22.442	99.045	4.413
PPL_07432	2.295	10.085	4.394
PPL_09669	4.501	19.681	4.372
PPL_07677	0.758	3.308	4.366
PPL_09729	4.441	18.623	4.194
PPL_05727	14.165	58.899	4.158
PPL_00342	8.880	36.626	4.125
PPL_04384	2.011	8.277	4.116
PPL_02977	0.240	0.954	3.978
PPL_00612	136.175	538.871	3.957
PPL_05027	7.782	30.380	3.904
PPL_12247	9.319	36.305	3.896
PPL_07802	1.955	7.609	3.892
PPL_04550	3.574	13.362	3.739
PPL_08725	12.027	44.048	3.662
PPL_09019	0.312	1.127	3.612
PPL_01689	5.176	18.299	3.535
PPL_01722	4.195	14.662	3.495
PPL_05544	11.525	38.768	3.364
PPL_04108	6.79	22.55	3.32
PPL_02831	0.177	0.583	3.296
PPL_11654	2.645	8.474	3.203
PPL_04393	5.199	16.529	3.179
PPL_07675	0.675	2.138	3.167
PPL_10323	1.282	3.905	3.045
PPL_00902	70.05	192.55	2.74
PPL_00855	1.72	2.99	1.48
PPL_02736	5.416	1.685	0.311
PPL_03602	0.516	0.158	0.306
PPL_00884	18.489	5.541	0.300
PPL_05333	15.452	4.574	0.296
PPL_10516	0.362	0.106	0.294
PPL_04954	2.062	0.594	0.288
PPL_06209	93.080	26.561	0.285
PPL_08648	10.447	2.953	0.283
PPL_10331	3.262	0.916	0.281
PPL_11619	304.579	83.717	0.275
PPL_03325	7.774	2.068	0.266
PPL_00578	16.604	4.376	0.264
PPL_03341	3.669	0.964	0.263
PPL_04828	3.315	0.848	0.256
PPL_06621	5.227	1.333	0.255
PPL_04238	0.616	0.155	0.251
PPL_02308	13.194	3.292	0.250
PPL_00897	1.786	0.440	0.246
PPL_07908	163.753	40.183	0.245
PPL_08647	19.482	4.674	0.240
PPL_09119	22.458	5.302	0.236
PPL_12443	15.613	3.634	0.233
PPL_01209	5.506	1.276	0.232

PPL_10531	0.874	0.201	0.230
PPL_02260	205.156	46.593	0.227
PPL_08055	11.058	2.505	0.227
PPL_12441	0.297	0.067	0.226
PPL_00231	2.668	0.596	0.223
PPL_07208	1.653	0.361	0.218
PPL_02829	20.565	4.274	0.208
PPL_03342	47.961	9.613	0.200
PPL_01921	0.971	0.193	0.199
PPL_02939	2.178	0.431	0.198
PPL_05195	69.666	13.699	0.197
PPL_08935	88.024	15.984	0.182
PPL_05491	1.091	0.195	0.179
PPL_01229	1.069	0.187	0.175
PPL_04307	1636.434	285.755	0.175
PPL_04306	1281.763	210.234	0.164
PPL_05306	0.323	0.049	0.151
PPL_05702	23.490	3.217	0.137
PPL_04465	6.978	0.900	0.129
PPL_05902	12.190	1.526	0.125
PPL_04042	37.560	3.818	0.102
PPL_05194	2.799	0.160	0.057

Table A5: List of genes regulated by glorin after *P. pallidum* PN500 cells prestarved for 1 hr were stimulated with glorin for 2 additional hours.

Gene ID	RPKM untreated	RPKM Glorin treated	Fold change treated/untreated
PPL_05354	15.810	1335.619	84.480
PPL_09347	0.264	21.669	82.061
PPL_12248	2.667	21.660	8.123
PPL_12249	8.729	67.574	7.741
PPL_00117	101.530	702.707	6.921
PPL_06644	0.587	3.173	5.408
PPL_00062	10.343	51.539	4.983
PPL_04459	4.801	22.411	4.668
PPL_12354	37.449	168.014	4.486
PPL_04587	2.970	13.157	4.430
PPL_05703	0.125	0.535	4.277
PPL_06407	6.709	27.641	4.120
PPL_02620	569.162	2267.988	3.985
PPL_06262	2.742	10.433	3.805
PPL_07551	13.510	47.156	3.491
PPL_10669	3.067	10.450	3.407
PPL_04328	0.114	0.389	3.405
PPL_06410	45.067	151.554	3.363
PPL_01619	68.645	228.550	3.329

PPL_11303	4.706	15.181	3.226
PPL_00902	96.22	148.75	1.54
PPL_00855	2.66	5.33	2.00
PPL_09762	31.752	10.371	0.327
PPL_02638	1.959	0.639	0.326
PPL_05154	11.139	3.539	0.318
PPL_04945	8.034	2.493	0.310
PPL_03339	34.276	10.616	0.310
PPL_04856	1.566	0.481	0.307
PPL_10751	3.645	1.108	0.304
PPL_11455	25.184	7.547	0.300
PPL_06963	5.062	1.478	0.292
PPL_08935	235.635	68.911	0.292
PPL_02856	140.397	40.876	0.291
PPL_03314	363.527	104.765	0.288
PPL_07479	27.195	7.770	0.286
PPL_04882	27.333	7.654	0.280
PPL_10745	39.311	10.956	0.279
PPL_11326	5.607	1.565	0.279
PPL_11401	32.889	9.128	0.278
PPL_08361	42.283	11.643	0.275
PPL_10576	212.746	57.775	0.272
PPL_07249	0.623	0.169	0.271
PPL_02535	0.790	0.212	0.269
PPL_04238	0.625	0.164	0.263
PPL_06004	8.414	2.213	0.263
PPL_06686	0.691	0.181	0.261
PPL_01117	5.524	1.423	0.258
PPL_05486	4.564	1.168	0.256
PPL_01872	1.090	0.279	0.255
PPL_03581	2.313	0.586	0.254
PPL_03364	1.715	0.426	0.248
PPL_04306	1049.866	259.797	0.247
PPL_04071	1.920	0.472	0.246
PPL_01230	39.811	9.723	0.244
PPL_02076	2.759	0.674	0.244
PPL_08030	0.297	0.072	0.243
PPL_04307	1296.892	314.696	0.243
PPL_00769	7.213	1.752	0.243
PPL_11952	0.652	0.158	0.243
PPL_11665	0.416	0.100	0.241
PPL_06751	0.575	0.139	0.241
PPL_05194	0.722	0.174	0.241
PPL_02626	0.416	0.100	0.241
PPL_02829	19.170	4.555	0.238
PPL_04785	40.309	9.566	0.237
PPL_06545	1.013	0.238	0.234
PPL_00136	72.238	16.741	0.232
PPL_08476	9.544	2.202	0.231
PPL_09772	0.110	0.025	0.230
PPL_00897	1.495	0.326	0.218
PPL_09119	19.644	4.283	0.218

PPL_04180	1.852	0.399	0.215
PPL_08475	10.241	2.171	0.212
PPL_07775	16.341	3.450	0.211
PPL_04561	1.008	0.213	0.211
PPL_12095	1.974	0.410	0.208
PPL_09571	0.491	0.101	0.207
PPL_08450	2.997	0.613	0.205
PPL_00658	2.774	0.563	0.203
PPL_10422	24.566	4.936	0.201
PPL_00038	0.162	0.032	0.199
PPL_11590	0.888	0.177	0.199
PPL_04774	1.921	0.372	0.194
PPL_08807	52.563	10.185	0.194
PPL_02624	5.276	1.019	0.193
PPL_07908	133.324	25.640	0.192
PPL_00514	93.525	17.746	0.190
PPL_05872	1.471	0.270	0.183
PPL_03343	3.365	0.606	0.180
PPL_00798	3.551	0.629	0.177
PPL_08488	15.832	2.809	0.177
PPL_12096	20.680	3.635	0.176
PPL_05902	47.110	8.213	0.174
PPL_01754	28.346	4.830	0.170
PPL_01394	10.409	1.723	0.166
PPL_08355	8.146	1.346	0.165
PPL_06552	74.177	12.011	0.162
PPL_01209	3.153	0.484	0.154
PPL_05156	8.090	1.214	0.150
PPL_06527	1.868	0.275	0.147
PPL_08477	50.321	7.318	0.145
PPL_02046	143.320	20.734	0.145
PPL_02260	242.138	34.257	0.141
PPL_02367	5.327	0.744	0.140
PPL_04465	10.235	1.371	0.134
PPL_07729	4.790	0.633	0.132
PPL_12441	0.418	0.054	0.130
PPL_03342	17.526	2.277	0.130
PPL_09005	35.352	4.452	0.126
PPL_08409	3.897	0.467	0.120
PPL_08976	20.505	2.367	0.115
PPL_00813	12.197	1.407	0.115
PPL_11308	14.053	1.583	0.113
PPL_00410	2.790	0.309	0.111
PPL_05333	32.013	3.080	0.096
PPL_03638	764.683	69.983	0.092
PPL_09004	12.918	1.177	0.091
PPL_07203	126.865	11.101	0.087
PPL_00519	2.116	0.179	0.084
PPL_01229	1.455	0.110	0.076
PPL_05195	117.677	8.034	0.068
PPL_07303	7.047	0.480	0.068

Table A6: Genes regulated by glorin after 1 hr of pre-starvation plus 1 or 2 hr of glorin treatment

Gene ID	Fold change 1 hr glorin-treated/untreated	Fold change 2 hr glorin-treated/untreated
PPL_05354	56.907	84.480
PPL_09347	53.915	82.061
PPL_12248	39.699	8.123
PPL_12249	29.237	7.741
PPL_06644	17.404	5.408
PPL_00117	12.128	6.921
PPL_02620	9.337	3.985
PPL_04587	7.610	4.430
PPL_11303	6.932	3.226
PPL_12354	6.332	4.486
PPL_10669	4.551	3.407
PPL_05333	0.296	0.096
PPL_04238	0.251	0.263
PPL_00897	0.246	0.218
PPL_07908	0.245	0.192
PPL_09119	0.236	0.218
PPL_01209	0.232	0.154
PPL_02260	0.227	0.141
PPL_12441	0.226	0.130
PPL_02829	0.208	0.238
PPL_03342	0.200	0.130
PPL_05195	0.197	0.068
PPL_08935	0.182	0.292
PPL_01229	0.175	0.076
PPL_04307	0.175	0.243
PPL_04306	0.164	0.247
PPL_04465	0.129	0.134
PPL_05902	0.125	0.174
PPL_05194	0.057	0.241

Table A7: GO term analysis of genes differentially regulated after 1 hour of pre-starvation plus 1 hr of glorin treatment.

PPL_00612	Molecular Function: catalytic activity (GO:0003824) Molecular Function: transcription repressor activity (GO:0016564) Molecular Function: binding (GO:0005488) Biological Process: metabolic process (GO:0008152)
PPL_00861	Molecular Function: protein kinase activity (GO:0004672) Molecular Function: ATP binding (GO:0005524) Molecular Function: protein serine/threonine kinase activity (GO:0004674) Biological Process: protein amino acid phosphorylation (GO:0006468)
PPL_02308	Molecular Function: ubiquitin-protein ligase activity (GO:0004842) Molecular Function: zinc ion binding (GO:0008270)
PPL_03541	Biological Process: pathogenesis (GO:0009405)
PPL_03564	Molecular Function: lipid binding (GO:0008289) Molecular Function: G-protein coupled receptor activity (GO:0004930), Biological Process: G-protein coupled receptor protein signaling pathway (GO:0007186) Cellular Component: integral to membrane (GO:0016021),
PPL_04042	Molecular Function: transcription factor activity (GO:0003700) Biological Process: regulation of transcription, DNA-dependent (GO:0006355) Cellular Component: transcription factor complex (GO:0005667)
PPL_04384	Molecular Function: signal transducer activity (GO:0004871) Molecular Function: two-component sensor activity (GO:0000155) Molecular Function: two-component response regulator activity (GO:0000156) Molecular Function: ATP binding (GO:0005524) Molecular Function: protein histidine kinase activity (GO:0004673) Molecular Function: two-component response regulator activity (GO:0000156) Molecular Function: transferase activity, transferring phosphorus-containing groups (GO:0016772) Biological Process: signal transduction (GO:0007165) Biological Process: two-component signal transduction system (phosphorelay) (GO:0000160) Biological Process: phosphorylation (GO:0016310) Biological Process: signal transduction (GO:0007165) Biological Process: regulation of transcription, DNA-dependent (GO:0006355) Biological Process: peptidyl-histidine phosphorylation (GO:0018106) Cellular Component: membrane (GO:0016020)
PPL_04393	Molecular Function: GTP binding (GO:0005525) Biological Process: small GTPase mediated signal transduction (GO:0007264) Cellular Component: intracellular (GO:0005622)

PPL_04465	Molecular Function: O-methyltransferase activity (GO:0008171)
PPL_04550	Molecular Function: glutamate-ammonia ligase activity (GO:0004356) Molecular Function: catalytic activity (GO:0003824) Biological Process: nitrogen compound metabolic process (GO:0006807)
PPL_04587	Cellular Component: viral capsid (GO:0019028)
PPL_05027	Molecular Function: ubiquitin thiolesterase activity (GO:0004221) Biological Process: ubiquitin-dependent protein catabolic process (GO:0006511)
PPL_05306	Molecular Function: O-methyltransferase activity (GO:0008171)
PPL_05333	Molecular Function: hydrolase activity, hydrolyzing O-glycosyl compounds (GO:0004553) Biological Process: carbohydrate metabolic process (GO:0005975)
PPL_05390	Molecular Function: deoxyribonuclease II activity (GO:0004531) Biological Process: DNA metabolic process (GO:0006259)
PPL_05392	Molecular Function: deoxyribonuclease II activity (GO:0004531) Biological Process: DNA metabolic process (GO:0006259)
PPL_05452	Molecular Function: GTP binding (GO:0005525) Biological Process: small GTPase mediated signal transduction (GO:0007264) Cellular Component: intracellular (GO:0005622)
PPL_05702	Biological Process: lipid metabolic process (GO:0006629) Biological Process: metabolic process (GO:0008152)
PPL_05727	Molecular Function: G-protein coupled receptor activity (GO:0004930) Molecular Function: lipid binding (GO:0008289) Molecular Function: GABA-B receptor activity (GO:0004965) Biological Process: G-protein coupled receptor protein signaling pathway (GO:0007186) Cellular Component: integral to membrane (GO:0016021)
PPL_06209	Molecular Function: hydrolase activity (GO:0016787) Molecular Function: sphingomyelin phosphodiesterase activity (GO:0004767), Biological Process: lipid metabolic process (GO:0006629) Biological Process: sphingomyelin catabolic process (GO:0006685)
PPL_06621	Molecular Function: catalytic activity (GO:0003824)
PPL_07296	Molecular Function: GTPase activity (GO:0003924) Molecular Function: GTP binding (GO:0005525) Biological Process: signal transduction (GO:0007165) Biological Process: small GTPase mediated signal transduction (GO:0007264) Cellular Component: membrane (GO:0016020) Cellular Component: intracellular (GO:0005622)
PPL_07302	Molecular Function: calcium ion binding (GO:0005509)
PPL_07432	Molecular Function: ATP binding (GO:0005524) Molecular Function: nucleotide binding (GO:0000166) Molecular Function: nucleoside-triphosphatase activity (GO:0017111) Molecular Function: ATPase activity (GO:0016887) Molecular Function: ATPase activity, coupled to

	transmembrane movement of substances (GO:0042626) Biological Process: transport (GO:0006810) Cellular Component: membrane (GO:0016020) Cellular Component: integral to membrane (GO:0016021)
PPL_07800	Molecular Function: protein binding (GO:0005515)
PPL_07812	Molecular Function: protein binding (GO:0005515)
PPL_07818	Molecular Function: protein binding (GO:0005515)
PPL_08055	Molecular Function: protein binding (GO:0005515)
PPL_08454	Molecular Function: lipid binding (GO:0008289) Molecular Function: G-protein coupled receptor activity (GO:0004930) Biological Process: G-protein coupled receptor protein signaling pathway (GO:0007186) Cellular Component: integral to membrane (GO:0016021)
PPL_08455	Molecular Function: lipid binding (GO:0008289) Molecular Function: G-protein coupled receptor activity (GO:0004930) Biological Process: G-protein coupled receptor protein signaling pathway (GO:0007186) Cellular Component: integral to membrane (GO:0016021)
PPL_08496	Biological Process: pathogenesis (GO:0009405)
PPL_08648	Biological Process: pathogenesis (GO:0009405)
PPL_08725	Molecular Function: protein binding (GO:0005515) Molecular Function: hydrolase activity (GO:0016787) Biological Process: metabolic process (GO:0008152)
PPL_09347	Molecular Function: actin binding (GO:0003779) Biological Process: cytoskeleton organization (GO:0007010) Biological Process: actin cytoskeleton organization (GO:0030036) Cellular Component: actin cytoskeleton (GO:0015629)
PPL_09669	Molecular Function: protein binding (GO:0005515) Molecular Function: zinc ion binding (GO:0008270) Cellular Component: intracellular (GO:0005622)
PPL_09729	Molecular Function: iron ion binding (GO:0005506) Molecular Function: oxidoreductase activity (GO:0016491) Molecular Function: oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen (GO:0016705) Molecular Function: L-ascorbic acid binding (GO:0031418) Biological Process: oxidation reduction (GO:0055114)
PPL_10331	Biological Process: steroid metabolic process (GO:0008202)
PPL_10669	Biological Process: pathogenesis (GO:0009405)
PPL_11619	Biological Process: metabolic process (GO:0008152) Molecular Function: oxidoreductase activity (GO:0016491)
PPL_12248	Molecular Function: protein serine/threonine kinase activity (GO:0004674) Molecular Function: ATP binding (GO:0005524) Biological Process: protein amino acid phosphorylation (GO:0006468)
PPL_12249	Molecular Function: protein serine/threonine kinase activity (GO:0004674) Molecular Function: ATP binding (GO:0005524) Biological Process: protein amino acid phosphorylation

	(GO:0006468)
PPL_12250	Molecular Function: protein serine/threonine kinase activity (GO:0004674) Molecular Function: ATP binding (GO:0005524) Biological Process: protein amino acid phosphorylation (GO:0006468)
PPL_12251	Molecular Function: protein serine/threonine kinase activity (GO:0004674) Molecular Function: ATP binding (GO:0005524) Biological Process: protein amino acid phosphorylation (GO:0006468)
PPL_12271	Molecular Function: MAP kinase activity (GO:0004707) Molecular Function: protein serine/threonine kinase activity (GO:0004674) Molecular Function: protein kinase activity (GO:0004672) Molecular Function: ATP binding (GO:0005524) Biological Process: protein amino acid phosphorylation (GO:0006468)
PPL_12443	Molecular Function: DNA binding (GO:0003677)

Table A8: GO term analysis of genes differentially regulated after 1 hour of pre-starvation plus 2 hours of glorin treatment.

PPL_00062	Molecular Function: binding (GO:0005488)
PPL_00136	Molecular Function: DNA binding (GO:0003677) Biological Process: nucleosome assembly (GO:0006334) Cellular Component: nucleosome (GO:0000786) Cellular Component: nucleus (GO:0005634)
PPL_00798	Molecular Function: sulfotransferase activity (GO:0008146) Biological Process: carbohydrate biosynthetic process (GO:0016051) Cellular Component: integral to membrane (GO:0016021)
PPL_00813	Molecular Function: signal transducer activity (GO:0004871)
PPL_01117	Molecular Function: flavin-containing monooxygenase activity (GO:0004499) Molecular Function: FAD binding (GO:0050660) Molecular Function: NADP or NADPH binding (GO:0050661) Biological Process: oxidation reduction (GO:0055114) Cellular Component: intrinsic to endoplasmic reticulum membrane (GO:0031227)
PPL_01230	Molecular Function: hydrolase activity, acting on ester bonds (GO:0016788)
PPL_01394	Molecular Function: transporter activity (GO:0005215) Biological Process: transport (GO:0006810) Cellular Component: cytoplasm (GO:0005737) Cellular Component: integral to membrane (GO:0016021)
PPL_01619	Molecular Function: zinc ion binding (GO:0008270) Molecular Function: hydrolase activity (GO:0016787) Molecular Function: catalytic activity (GO:0003824)
PPL_02046	Molecular Function: triglyceride lipase activity (GO:0004806) Biological Process: lipid metabolic process (GO:0006629)
PPL_02535	Molecular Function: protein binding (GO:0005515)
PPL_02856	Molecular Function: triglyceride lipase activity (GO:0004806) Biological Process: lipid metabolic process (GO:0006629)
PPL_03364	Cellular Component: chromosome, centromeric region (GO:0000775), Molecular Function: DNA binding (GO:0003677)
PPL_04071	Molecular Function: monooxygenase activity (GO:0004497) Molecular Function: iron ion binding (GO:0005506) Molecular Function: electron carrier activity (GO:0009055) Molecular Function: heme binding (GO:0020037) Biological Process: oxidation reduction (GO:0055114)
PPL_04328	Molecular Function: deoxyribonuclease II activity (GO:0004531) Biological Process: DNA metabolic process (GO:0006259)
PPL_04465	Molecular Function: O-methyltransferase activity (GO:0008171)
PPL_04587	Cellular Component: viral capsid (GO:0019028)
PPL_05154	Biological Process: metabolic process (GO:0008152) Molecular Function: oxidoreductase activity (GO:0016491)
PPL_05156	Molecular Function: O-methyltransferase activity (GO:0008171)
PPL_05333	Molecular Function: hydrolase activity, hydrolyzing O-glycosyl compounds (GO:0004553) Biological Process: carbohydrate metabolic process

	(GO:0005975)
PPL_05703	Molecular Function: carbohydrate binding (GO:0030246) Cellular Component: extracellular region (GO:0005576)
PPL_06262	Biological Process: pathogenesis (GO:0009405)
PPL_06545	Biological Process: sphingolipid metabolic process (GO:0006665) Biological Process: lipid metabolic process (GO:0006629) Cellular Component: lysosome (GO:0005764)
PPL_06552	Biological Process: pathogenesis (GO:0009405)
PPL_06686	Molecular Function: zinc ion binding (GO:0008270) Cellular Component: intracellular (GO:0005622)
PPL_07249	Molecular Function: catalytic activity (GO:0003824) Molecular Function: oxidoreductase activity (GO:0016491) Molecular Function: binding (GO:0005488) Biological Process: metabolic process (GO:0008152)
PPL_07551	Molecular Function: ATP binding (GO:0005524) Molecular Function: ATPase activity (GO:0016887) Molecular Function: nucleotide binding (GO:0000166) Molecular Function: nucleoside-triphosphatase activity (GO:0017111)
PPL_07729	Biological Process: sphingolipid metabolic process (GO:0006665) Biological Process: lipid metabolic process (GO:0006629) Cellular Component: lysosome (GO:0005764)
PPL_08355	Molecular Function: hydrolase activity, hydrolyzing O-glycosyl compounds (GO:0004553) Biological Process: carbohydrate metabolic process (GO:0005975)
PPL_08361	Molecular Function: GTP binding (GO:0005525), Biological Process: small GTPase mediated signal transduction (GO:0007264)
PPL_08450	Biological Process: pathogenesis (GO:0009405) Molecular Function: sugar binding (GO:0005529)
PPL_08475	Biological Process: pathogenesis (GO:0009405)
PPL_08476	Biological Process: pathogenesis (GO:0009405)
PPL_09347	Molecular Function: actin binding (GO:0003779) Biological Process: cytoskeleton organization (GO:0007010) Cellular Component: actin cytoskeleton (GO:0015629)
PPL_09571	Molecular Function: acid phosphatase activity (GO:0003993) Molecular Function: metal ion binding (GO:0046872) Molecular Function: hydrolase activity (GO:0016787
PPL_09762	Molecular Function: ATP binding (GO:0005524) Biological Process: protein import (GO:0017038) Cellular Component: membrane (GO:0016020)
PPL_10669	Biological Process: pathogenesis (GO:0009405)
PPL_11326	Molecular Function: protein serine/threonine kinase activity (GO:0004674) Molecular Function: protein kinase activity (GO:0004672) Molecular Function: ATP binding (GO:0005524) Molecular Function: ATP binding (GO:0005524) Biological Process: protein amino acid phosphorylation (GO:0006468)

PPL_11401	Molecular Function: cysteine-type endopeptidase activity (GO:0004197) Molecular Function: cysteine-type peptidase activity (GO:0008234) Biological Process: proteolysis (GO:0006508) Cellular Component: extracellular region (GO:0005576)
PPL_12248	Molecular Function: protein serine/threonine kinase activity (GO:0004674) Molecular Function: ATP binding (GO:0005524) Biological Process: protein amino acid phosphorylation (GO:0006468)
PPL_12249	Molecular Function: protein serine/threonine kinase activity (GO:0004674) Molecular Function: ATP binding (GO:0005524) Biological Process: protein amino acid phosphorylation (GO:0006468)

Table A9: Complete List of GPCR genes upregulated by glorin

Cells were staved for 2 or 3 hours without glorin treatment (t2, t3) or pre-starved for 1 hour and treated with glorin for additional 1 or 2 hours (t2+, t3+). Fold changes are shown that were obtained by comparing gene expression in glorin-treated cells versus untreated cells.

Locus in genome	Gene Name	t2+/t2	t3+/t3	Putative Identification (IPR description)	Orthologs
PPL_03884	grlE	1.20755454	1.08147317	G-protein-coupled receptor (GPCR) family 3 protein 5, GABA-B receptor protein G-protein coupled receptor activity (GO:0004930) GABA-B receptor activity (GO:0004965) triacylglycerol lipase activity (GO:0004806)	*D.discoideum/ D.fasciculatum*
PPL_00902	grlL	2.74841933	1.545949343	G-protein-coupled receptor (GPCR) family 3 protein 11, lipid binding (GO:0008289) G-protein coupled receptor activity (GO:0004930) GABA-B receptor activity (GO:0004965)	*D.discoideum/ D.fasciculatum*
PPL_09248	grlF	0.825961763	0.959042267	G-protein-coupled receptor (GPCR) family 3 protein 6 lipid binding (GO:0008289) G-protein coupled receptor activity (GO:0004930)	
PPL_05727	grlH	4.158168581	1.038539037	G-protein-coupled receptor (GPCR) family 3 protein 8 lipid binding (GO:0008289) G-protein coupled receptor activity (GO:0004930) GABA-B receptor activity (GO:0004965	*D.discoideum/ D.fasciculatum*
PPL_03564		8.65643913	1.696856519	G-protein-coupled receptor (GPCR) family 3 protein lipid binding (GO:0008289) G-protein coupled receptor activity (GO:0004930)	*D.discoideum/ D.fasciculatum*
PPL_10637	grlM	1.180710312	0.931420984	G-protein-coupled receptor (GPCR) family 3 protein 12 G-protein coupled receptor activity (GO:0004930) lipid binding (GO:0008289)	*D.discoideum/ D.fasciculatum*
PPL_05022	grlD	0.830265822	1.749651374	G-protein-coupled receptor (GPCR) family 3 protein 4 GABA-B receptor activity (GO:0004965) lipid binding (GO:0008289) G-protein coupled receptor activity (GO:0004930)	*D.discoideum/ D.fasciculatum*
PPL_04108	grlC	3.321081894	0.929070865	lipid binding (GO:0008289), G-protein coupled receptor activity (GO:0004930)	*D.discoideum/ D.fasciculatum*
PPL_08454		15.86575363	2.66083276	G-protein-coupled receptor (GPCR) family 3 protein G-protein coupled receptor activity (GO:0004930) lipid binding (GO:0008289)	*D.discoideum/ D.fasciculatum*
PPL_08455	Metabotropic glutamate	11.98277423	2.021828818	G-protein-coupled receptor (GPCR) family 3 protein, G-protein coupled	*D.discoideum*

	receptor-like protein			receptor activity (GO:0004930) lipid binding (GO:0008289)	
PPL_05762	grlR	0.789895954	0.800592227	G-protein-coupled receptor (GPCR) family 3 protein 17, G-protein coupled receptor activity (GO:0004930)	*D.discoideum/ D.fasciculatum*
PPL_09437		0.416878904	0.667195531	G-protein-coupled receptor (GPCR) family 3 protein G-protein coupled receptor activity (GO:0004930)	*D.discoideum/* *D.fasciculatum*
PPL_05381	crlF	1.047911068	0.977542476	GPCR, family 2-like transmembrane receptor activity (GO:0004888)	*D.discoideum/ D.fasciculatum*
PPL_10136	crlC	1.632033321	0.644915034	cAMP receptor-like protein, G-protein-coupled receptor (GPCR) family protein GPCR, rhodopsin-like superfamily GPCR, family 2-like transmembrane receptor activity (GO:0004888)	*D.discoideum/ D.fasciculatum*
PPL_01693	carB	0.47333985	0.665297373	G-protein-coupled receptor, cAMP receptor 2, G-protein coupled receptor activity (GO:0004930), cAMP binding (GO:0030552) transmembrane receptor activity (GO:0004888)	*D.discoideum/ D.fasciculatum*
PPL_05277		1.148618881	0.936387653	G-protein coupled receptor activity (GO:0004930), cAMP binding (GO:0030552) transmembrane receptor activity (GO:0004888) rhodopsin-like superfamily GPCR, family 2-like	*D.discoideum/ D.fasciculatum*
PPL_00085		0.640793725	1.192893791	cAMP-type GPCR GPCR, family 2-like G-protein coupled receptor activity (GO:0004930), cAMP binding (GO:0030552) transmembrane receptor activity (GO:0004888)	*D.discoideum/ D.fasciculatum*
PPL_07940	crlA	0.782860038	0.790505126	G-protein coupled receptor activity (GO:0004930), cAMP binding (GO:0030552) Cellular component: integral to membrane (GO:0016021)	*D.discoideum/ D.fasciculatum*
PPL_08101	crlE	1.119936881	0.73910168	GPCR, family 2-like transmembrane receptor activity (GO:0004888)	*D.discoideum/ D.fasciculatum*
PPL_06402	cAMP-type GPCR	1.30693551	1.126307933	G-protein coupled receptor activity (GO:0004930), cAMP binding (GO:0030552) transmembrane receptor activity (GO:0004888)	*D.discoideum/ D.fasciculatum*
PPL_03211		0.897006864	0.78965748	GPCR, family 2-like cAMP-type GPCR secretin-like receptor, latrophilin receptor-like protein, transmembrane receptor activity (GO:0004888) G-protein coupled receptor activity (GO:0004930), cAMP binding (GO:0030552)	*D.discoideum/ D.fasciculatum*
PPL_11745	fscC	0.378863325	0.416001857	G-protein-coupled receptor (GPCR) family protein, frizzled and smoothened-like sans CRD protein, transmembrane receptor activity (GO:0004888)	*D.discoideum/ D.fasciculatum*
PPL_09871	fslQ	1.030847142	0.773862415	G-protein-coupled receptor (GPCR) family protein, frizzled and smoothened-like protein	*D.discoideum/ D.fasciculatum*

				transmembrane receptor activity (GO:0004888)	
PPL_11273	fscH	1.58459391	1.512767255	G-protein-coupled receptor (GPCR) family protein, frizzled and smoothened-like sans CRD protein, GPCR, family 2-like transmembrane receptor activity (GO:0004888)	*D.discoideum/ D.fasciculatum*
PPL_02857		1.188110538	1.158026463	ARF GTPase activator activity (GO:0008060), zinc ion binding (GO:0008270) transmembrane receptor activity (GO:0004888)	D.discoideum/ D.fasciculatum
PPL_00855		1.48	1.89	G-protein-coupled receptor (GPCR) family protein, frizzled and smoothened-like sans CRD protein	D.discoideum/ D.fasciculatum

List of scientific publications and presentations

Asghar A, Groth M, Siol O, Gaube F, Enzensperger C, Glöckner G, Winckler T. (2012) Developmental gene regulation by an ancient intercellular communication system in social amoebae. *Protist.* 163(1):25-37.

Asma Asghar, Gernot Glöckner, Oliver Siol, Thomas Winckler (2010).
Glorin-mediated gene expression in *Polysphondylium pallidum*. The 2010 International Dictyostelium Symposium held in Cardiff, Wales, UK, Aug^{1st}- Aug 6th, 2010

Asma Asghar, Marco Groth, Oliver Siol, Thomas Winckler (2010).
Preliminary characterization of peptide-based communication in social amoebae. The 2010 International Dictyostelium Symposium held in Cardiff, Wales, UK, Aug^{1st}- Aug 6th, 2010

Asma Asghar, Gernot Glöckner, Marco Groth, Thomas Winckler (2009)
Initial characterization of glorin-mediated gene expression in *Polysphondylium pallidum.* The 2009 International Dictyostelium Symposium held in Estes, Colorado, USA, August 23rd -28th, 2009.

Asma Asghar, Christoph Enzensperger, Thomas Winckler (2010).
Roots of peptide-based communication in social amoebae. The 2010 MiCom conference on microbial interactions held in Jena, Germany. Sep 28 th - October 2nd , 2010

Asma Asghar, Gernot Glöckner, Christoph Enzensperger, Thomas Winckler (2009)
Exploring glorin-mediated cell-cell communication in *Polysphondylium pallidum*. The 2009 JSMC annual symposium held in Jena, Germany. October 11th-12th, 2009.

Asma Asghar & Thomas Winckler (2008)
Intercellular communication with peptide derivatives in social amoebae. The 2008 JSMC annual symposium held in Jena, Germany. December 14th -15th, 2009.

Acknowledgements

The presented research work was completed between October 2008 and October 2011 at the Department of Pharmaceutical Biology, Institute of Pharmacy, Friedrich Schiller University Jena, Germany. I would like to thank all the people mentioned below who contributed to the success of this project.

First and foremost, I express my heartfelt gratitude and indebtedness to my supervisor Prof. Dr. Thomas Winckler for providing me an opportunity to work under his esteemed supervision. I am grateful to him for his invaluable guidance, creative suggestions and inspiring enthusiasm for science that helped me to develop my scientific skills. I admire the dedication and passion he has for great research. I am thankful to him for being instrumental in planning and shaping up my thesis in an exceptional way; without his superb guidance I stand nowhere in this rapidly growing world of science. Words cannot replace my special thanks for his immense moral support and encouragement when I was struggling between the hard core of my life and the dream to have my PhD degree.

I am deeply indebted to Prof. Dr. Christian Hertweck (Biomolecular chemistry group, Hans Knöll Institute, Jena), my co-supervisor who was always ready to offer help, guidance and constructive ideas. His direction, assistance and intellectually stimulating discussions have further advanced my appreciation for science in general.

I would especially like to thank Dr. Gernot Glöckner (Berlin Centre for Genomics in Biodiversity research, Berlin) for sharing genome sequencing data of *P. pallidum* PN500 before it was published.

I would also like to acknowledge Prof. Dr. Pauline Schaap (who is a role model for me) and Dr. Christina Schilde from the Univeristy of Dundee, Scotland for their intellectual discussions, fascinating ideas and sharing lab materials and protocols.

I pay my special thanks to Dr. Maria Romerlao and Dicty Stock Center for providing *Dictyostelid* strains.

I would like to forward my thanks to Dr. Karl-Heinz Gührs (Proteomics facility- Fritz Lipmann Institute Jena, Germany) for helping me in proteome analysis, Dr. Marco Groth (Genome analysis group; Fritz Lipmann Institute Jena) for conducting RNA sequencing (RNA-seq), Dr. Oliver Siol

for RNA-seq data analysis, and Dr. Christoph Enzensperger (Institute of Pharmaceutical Chemistry, University of Jena) for synthesizing glorin at Institute of Pharmaceutical chemistry.

I would like to thank the team of incredible researchers that I have spent the past three years with. Dr. Friedemann Gaube has been a wonderful colleague, who always extended his help whenever I approached him throughout my stay at the Institute of Pharmaceutical Biology. The fellow PhD scholars, Jana, Anika, Sara, Thomas, Michael and Tilmann have been fun to work with and I have enjoyed being part of such a friendly team. Bärbel, Heide, and Angela, I can never forget your affection!

I would like to acknowledge Jena School for Microbial Communication (JSMC) for offering me a PhD position and supporting my scientific career. It has contributed greatly to the development of my personality as a young researcher. I extend my special and sincere gratitute to Dr. Carsten Thoms (manager JSMC) and Frau Ulrike Schleier (secretary JSMC) for being so supportive, encouraging, friendly and tolerant.

My acknowledgements would be highly incomplete if I don't mention some nice friends; Stefanie, Chithra Devi and her husband Monickam, Mona (my neighbour), Hanadi, Bushra, Wafa, Karoline, Bita, Alex, and Thobias Koch who always showed me a positive outlook towards life and made my time lively.

Last but not the least, I would like to appreciate the incredible love and support of my family who have always been there and have been the drive that kept me going, always taking interest in the work I have completed. I deeply acknowledge their patience, tolerance, practical advices and sacrifices that boosted me towards my success. A special note of thanks to Ammi, Abo, Shummaila Baji, Shummail Bhai, Yasir (he has been my best childhood friend though we argue alot) and his wife Ana, Bilal, Shani and Usama (for his innocent love towards me). Thanks to all the kids also; Nimrah, Airah, Muaaz, Iman and Mahnoor.

Asma ASGHAR

30 July, 2012
Jena, Germany

Lebenslauf

Name, Vorname:	Asma Asghar
Adresse:	Schlegelstr. 6, D-07747 Jena
Geburtsdatum:	08.02.1982
Nationalität:	Pakistanisch
Schulausbildung / Ausbildung:	
1986 – 1996	Nusrat Gymnasium Al-Faisal Town Lahore, Pakistan
1997 – 1999	Pre- medizinische Studien Lahore Universität für Frauen Lahore, Pakistan
Universitätsstudium:	
2000 – 2004	Bachelor der Pharmazie Institut der Pharmazie Universität Punjab, Lahore, Pakistan
2005– 2007	Master der Molekularbiologie Nationales Zentrum der Molekularbiologie Lahore, Pakistan
2007 – 2008	Forschungsassistentin Institut der Biochemie Technische Universität Graz, Österreich
Promotionsstudium:	
2008 – 2012	PhD Scholar Lehrstuhl für pharmazeutische Biologie Institut der Pharmazie Friedrich Schiller Universität Jena, Deutschland

Curriculum Vitae

Name:	Asma Asghar
Address:	Schlegelstr. 6, D-07747 Jena
Date of birth:	08.02.1982
Nationality:	Pakistani
School & College Education:	
1986 – 1996	Nusrat High School Al-Faisal Town Lahore Cantt, Pakistan
1997 – 1999	Pre- Medical Studies Lahore College for Women Lahore, Pakistan
University studies:	
2000 – 2004	Bachelor of Pharmacy Institute of Pharmacy University of the Punjab, Lahore, Pakistan
2005– 2007	Master of Molecular Biology National Centre of Excellence in Molecular Biology Lahore, Pakistan
2007 – 2008	Research Assistant Institute of Biochemistry Graz University of Technology, Graz, Austria
Doctoral Studies:	
2008 – 2012	PhD Scholar Department of Pharmaceutical Biology Institute of Pharmacy Friedrich Schiller University Jena Germany

Ehrenwörtliche Erklärung

Hiermit erkläre ich,

- dass mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät bekannt ist,
- dass ich die Dissertation selbst angefertigt habe, keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen worden sind und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,
- dass ich die Hilfe eines Promotionsberaters nicht in Anspruch genommen habe und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,
- dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe
- dass ich die vorliegende Arbeit weder komplett noch in Form einer in wesentlichen Teilen ähnlichen oder anderen Abhandlung bei der Friederich-Schiller-Universität oder einer anderen Hochschule als Dissertation oder Prüfungsarbeit für eine staatliche oder wissenschaftliche Prüfung vorgelegt habe.

Jena, den 30 Juli 2012

Asma Asghar